THE CARBOHYDRATE CONSTITUENTS OF GRASSES AND CLOVERS.

A Thesis submitted for the degree of Doctor of Philosophy

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Introduction

Plants may be divided into two classes,

(i) the flowerless - the cryptogams e.g. ferns, seaweeds, mosses, and

(ii) the flowering - the phanerogams i.e. the seed-bearing plants.

The latter class has been further divided into two groups

(i) the gymnosperms which have their seeds uncovered e.g. the coniferous plants, and

(ii) the angiosperms whose seeds are enclosed.

By far the greater number of the phanerogams belong to the angiosperm group and botanists have divided it for convenience into subgroups - the monocotyledons, which bear only one seed leaf, and the dicotyledons which possess two seed leaves. The grasses (Gramineae) are examples of the former group, while the clovers and lucerne belong to the latter and differences in the chemical composition of the two groups will be shown in this thesis.

The carbohydrates which occur in plants can be divided into (i) the water-soluble sugars i.e. mono-, di-saccharides, oligosaccharides of short chain length, and a reserve polysaccharide,

and (ii) the cell-wall polysaccharides i.e. the cellulose and the hemicelluloses which are large complex molecules whose detailed structures are in most cases not yet known.

Although much analytical work has been carried out on the carbohydrate constituents of herbage the results from the methods used have produced little evidence about the processes taking place in the living plant. For routine analysis of herbage, it was necessary to use methods which could readily be applied to a large number of samples. With the older methods of carbohydrate analysis, this meant that obtaining detailed information about the changes in the mono-, di-, and oligo-saccharides became a very formidable if not impossible task, and this hindered any major advances in fundamental investigations.

The early procedure had been to divide the carbohydrates into two fractions (a) the crude fibre, and (b) the nitrogen-free-extractives.

The crude fibre represents that portion of the plant material which remains insoluble after the plant has been extracted with water or aqueous ethanol, and then subjected to the action of a dilute mineral acid for a short time. This residue may account for 30% of the dry matter of the herbage. Norman (99) pointed out in 1935 that the crude fibre was not a single chemical compound, but consisted of cellulose, lignin and hemicelluloses occurring in different ratios in various plant species. A typical

hay crude fibre (98) was found to consist of cellulose 80.1%, lignin 11.5, pentosans 10.9 (a mixture of araban and xylan) and a small amount of protein and ash.

The nitrogen-free-extractives (N.F.E.) represent the portion of the plant remaining unaccounted for after the crude protein, crude fibre, ash, ether extractives have been estimated. From the method of its determination, it is to be expected that its composition would be variable. One example (98) showed the N.F.E. to include cellulose 20.5%, lignin 9.7, pentosans 29.4. The remaining 40.4% are mainly water-soluble sugars, hexosans and organic acids.

These two fractions, although they do include all the carbohydrate components, can give no information about changes in the amount of any individual carbohydrate constituents since the crude fibre contained 17.7% of the pentosans and 69.3%of the cellulose, the remaining 82.3% and 30.7% occurring in the N.F.E.

The more recent methods for the analysis of the water-soluble sugars, e.g. that described by Waite and Boyd (135), have been based on the determination of the reducing sugars in alcohol and water extracts before and after acid hydrolysis. In some cases, fructose has been estimated after oxidation of glucose in mixtures of these two sugars. Such methods possess two disadvantages; (a) small quantities of reducing substances other than carbohydrates will be included, and (b) the sucrose value may not represent only the sucrose present but will also include oligosaccharides.

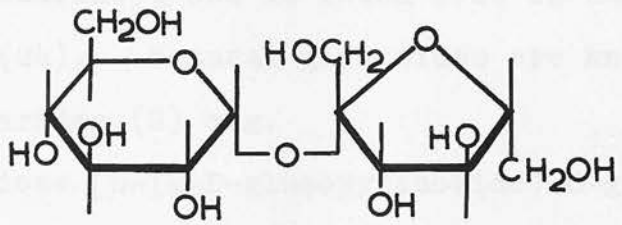
With the development of chromatography and its application to carbohydrate analysis, it has been possible to devise routine methods for the detailed analysis of plant materials. This thesis contains the results of analyses of grass and lucerne using these new techniques. The amounts of glucose, fructose, sucrose and oligosaccharides in these plants have been accurately determined at intervals during the growth cycles and the simultaneous changes in the amount of reserve carbohydrate and cell-wall polysaccharides have been observed. From the results it has been possible to understand more fully the changes occurring during the plant's growth.

Synthesis in the plant is a photochemical reaction in which the carbon dioxide of the atmosphere and the water of the plant are converted into carbohydrate using the energy of sunlight. The reaction is complex, but sucrose, glucose and fructose are the first sugars to accumulate in any great quantity.

Glucose and fructose are the only common monosaccharides found uncombined in grasses and clovers although others have been reported in other plants. Rhamnose was detected in an ether extract of the leaves of poison ivy early this century (1) but it is probable that this arose from the degradation of a glycoside. The heptose D-mannoheptulose has been found in avocado pears (78), and D-sedoheptulose (111,79) in Sedum spectabile and in primulae. Recent work by Benson, Bassham and

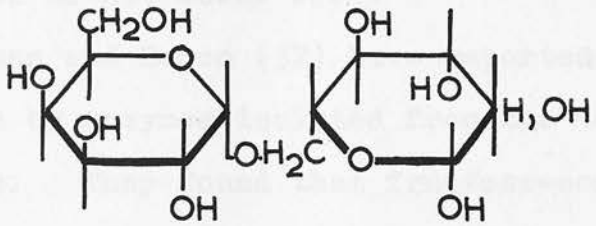
Calvin has demonstrated that sedoheptulose takes part in the photosynthetic cycle (15) and these authors suggest that the free sugar may be liberated enzymatically from its phosphate during the killing of the plant. The ketopentose ribulose as the diphosphate also plays an important part in photosynthesis where it is thought to function as the carbon dioxide acceptor in the cycle (143).

Of the disaccharides found naturally, sucrose [α-D-glucopyranosido-β-D-fructofuranoside]



is by far the most abundant.

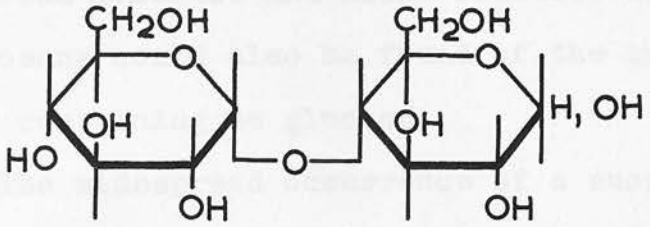
Melibiose [6-(α-D-galactopyranosido)-D-glucopyranose]



is thought to occur free in perennial rye grass (Lolium perenne)(82) and an early report (25) claimed its presence free in wild mallow, although it is possible that in both instances it may have arisen

by loss of a fructose residue from raffinose during isolation.

Maltose [4-(α-D-glucopyranosido)-D-glucopyranose]



may possibly be present in plants which contain starch (this disaccharide may be regarded as a structural unit for that polysaccharide), and is found free in small quantities in barley grains (24). Natural glycosides are known to contain other disaccharides (2) e.g.
gentiobiose [6-(β-D-glucopyranosido)-D-glucose],
rutinose [6-(β-L-rhamnopyranosido)-D-glucose],
primverose [6-(β-D-xylopyranosido)-D-glucose], and
vicianose [6-(α-L-arabinopyranosido)-D-glucose] but the sugar units do not occur free.

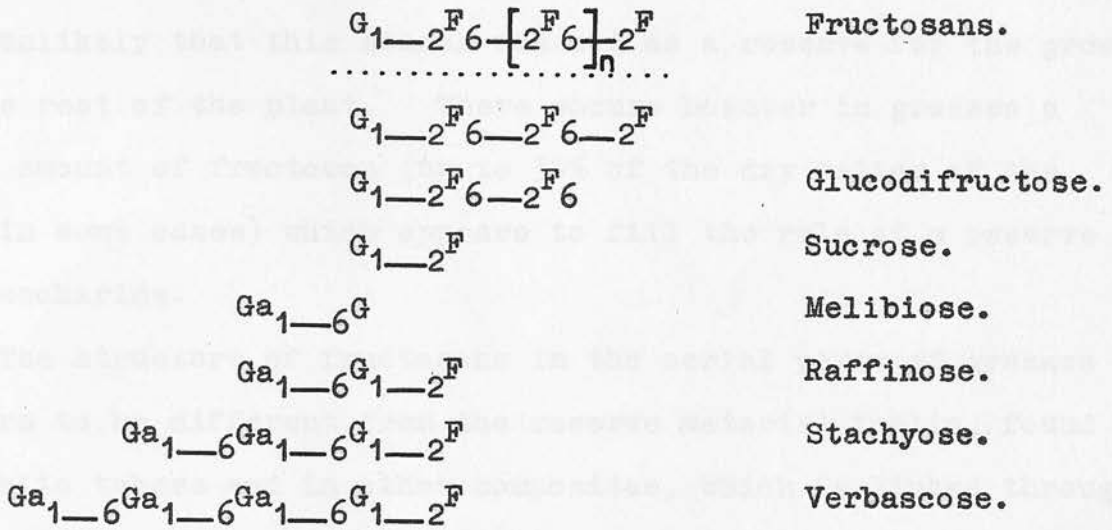
Edelman and Bacon (37) have reported the synthesis of fructosans by enzymes isolated from the tubers of Jerusalem artichokes. They found that fructose-containing oligosaccharides were built up by transfructosylation from inulin to fructose, sucrose, raffinose and melezitose. Prolonged incubation (with for example raffinose) gave rise to higher oligosaccharides.

These findings were confirmed independently by Dedonder (35). Such reactions would account for the presence of glucose in the fructosan molecule and where fructose is the initial substrate, fructosans could also be found of the type reported by Schlubach (115) containing no glucose.

The widespread occurrence of a sucrose unit in the fructosans and in the raffinose family of oligosaccharides (52) indicates its importance in the plant world where it occurs as one of the earliest products which can be isolated from the photosynthetic reactions.

The trisaccharide raffinose (82,150) and the tetrasaccharide stachyose (82) are thought to be present in perennial ryegrass, and it is not unusual to find series of oligosaccharides of increasing chain lengths in plants (37,69). For instance MacLeod has found a series glucodifructose to glucopentafructose in barley grains (89) and Harwood, Laidlaw and Telfer have found in perennial ryegrass a series of fructosans of low molecular weight having chain lengths five to ten units (62). It appeared likely that these chains were terminated by glucopyranose units linked as in sucrose. The inter-relation of glucose, fructose, sucrose, raffinose, melibiose

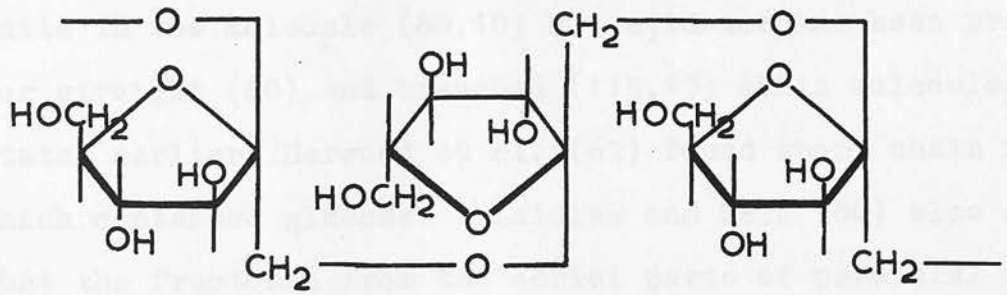
and the fructosans is shown in the following figure.



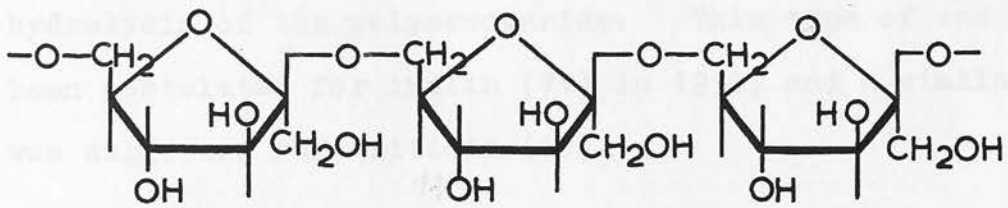
In addition to the smaller molecules found in grasses and clovers which can be extracted with 80% or 90% ethanol, and the cell-wall polysaccharides, there is normally a water-soluble polysaccharide. In the dicotyledons, starch is frequently found and its chief biological function is to act as a reserve carbohydrate. It was thought that no starch was present in clovers (82) and that the araban and galactan and possibly a water-soluble glucosan might function as reserve, however Hardwick (56) has detected a "starch-like" material in Subterranean clover which cannot be dissolved by the normal water extractions and following the precedent of McIlvanie (87) she has called it a "reserve polysaccharide." It gives a blue colour with iodine.

Starch does not usually appear in the monocotyledons except when laid down in the seed during its formation, and it would seem unlikely that this starch can act as a reserve for the growth of the rest of the plant. There occurs however in grasses a large amount of fructosan (up to 30% of the dry matter of the stem in some cases) which appears to fill the role of a reserve polysaccharide.

The structure of fructosans in the aerial parts of grasses appears to be different from the reserve material inulin, found in dahlia tubers and in other compositae, which is linked through carbon atoms numbers 1 and 2 in the molecule. Schlubach and Sinh (117) have divided fructosans into two groups, the inulin type having links between carbon atoms 1 and 2,



and the phlein type, having $C_2 - C_6$ linkages,

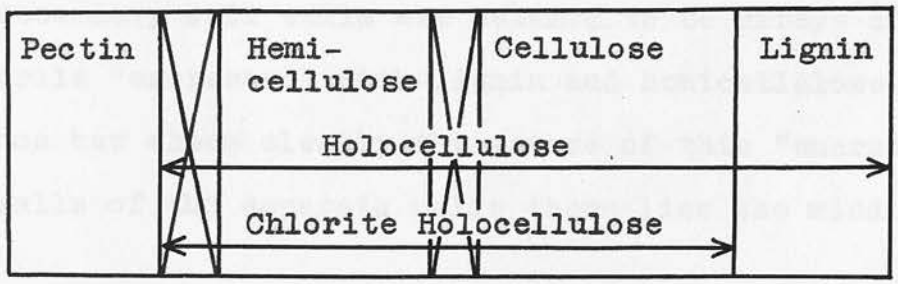


Fructosans of the latter type have been isolated from the aerial parts of many of the gramineae e.g. from Meadow Grass (Poa trivialis) (22), from barley leaves (63), from Italian Ryegrass (Lolium multiflorum) (13), from leafy cocksfoot (Dactylis glomerata) (13), from timothy (Phleum pratense) (117), from unripe rye (114), from wheat stalks (116) and from perennial ryegrass (80,115). The fructosan, triticin, isolated from the rhizomes of couch grass (Agropyron repens) (10) appears to be intermediate in composition between the inulin and phlein types, in that it contains C_1-C_2 and C_2-C_6 linkages in equal proportions.

It is unlikely that all these fructosans have the same structure. They appear to contain about thirty fructofuranose units in the molecule (80,10) but evidence has been presented for straight (80) and branched (115,13) chain molecules. As stated earlier, Harwood et al. (62) found short chain fructosans which contained glucose. Laidlaw and Reid (80) also showed that the fructosan from the aerial parts of perennial ryegrass gave on hydrolysis 98% fructose and 2% glucose, and Aspinall and Telfer (11) were able to isolate sucrose after partial hydrolysis of the polysaccharide. This type of end group had been postulated for inulin (71) in 1950, and a similar grouping was suggested for triticin (10).

Archbold (8) has reviewed very fully the fructosans occurring in monocotyledonous plants, discussing the preparation of the polysaccharides and their chemical constitution. The author also compares the changes found under abnormal growth conditions with the normal seasonal changes. For example nitrogen deficiency in barley plants gives rise to increased fructosan and alcohol-soluble sugar contents, while a potassium deficiency has the effect of lowering the amount of fructosan present. Mutilation of barley plants was also shown to affect the fructosan contents; for instance, tiller removal caused an increase in the total water-soluble sugar content in the rest of the plant.

In "Modern Methods of Plant Analysis", Jermyn (95a) emphasises that the terms cellulose and hemicellulose do not represent specific chemical entities but are the products obtained as a result of a series of chemical operations. The relation between holocellulose, hemicellulose and the other fractions encountered in polysaccharide chemistry is depicted in the following diagram,



The pectin is most readily removed, 0.5% Ammonium oxalate being the usual reagent for this action. The holocellulose includes the hemicellulose, cellulose and lignin but by treatment with acid chlorite, it is possible to remove the lignin, leaving a product known as the chlorite holocellulose. In many cases it is impossible to prepare this without degradation of the polysaccharides but in many chemical analyses, it is the chlorite holocellulose which is used as the starting material for study. The strength of the alkali used to extract the holocellulose determines how much of the hemicellulose is removed and by definition (95b), the α-cellulose is that portion of the chlorite holocellulose not dissolved by 24% potassium hydroxide (or 17.5% sodium hydroxide). The border zone between the cellulose and hemicellulose gives rise to some trouble in the interpretation of analytical results, since its position depends upon the conditions actually employed in the analysis.

Little is known about the organisation of the plant cell walls at the present time. A recent summary (110) from the biophysical viewpoint summarises the relevant literature. The primary and secondary cell walls are assumed to be arrays of cellulose fibrils "encrusted" with lignin and hemicellulose, but no evidence has shown clearly the nature of this "encrusting". Between the walls of the separate cells there lies the middle

lamella, composed of the complex of polysaccharides known as pectin.

The chemical constitution of these carbohydrate fractions is known only in rough detail. The α-cellulose consists of long chains of β-D-glucopyranose rings linked through carbon atoms 1 and 4, while the hemicellulose is believed to contain somewhat shorter chains containing xylose, mannose and uronic acids. As between the hemicellulose and cellulose there appears to be an indefinite boundary on the other side between hemicellulose and pectin. The pectin is composed of chains of galacturonic acid residues linked, it is thought, in a manner similar to the polysaccharide units, but there are associated with it, galactose and arabinose residues and it is these which give rise to the broad separation. It is not known whether these carbohydrates, which are associated closely with pectin, should be classified with the pectin fraction or whether they should be included in the hemicellulose.

The basis of the classification of the polysaccharides used in the course of this present work has been as follows. The pectin was regarded as being the polygalacturonic acid material and the arabinose and galactose has been included in the hemicellulose along with all the xylose and mannose (in lucerne) found in the plant. The cellulose represents the amount of glucose material present in the polysaccharides.

The complex nature of the polysaccharides is shown by the inability of chemists to prepare pure fractions from the hemicellulose isolated from cocksfoot grass. The earlier work (by Buston [20]) yielded only five fractions all containing different amounts of xylose and arabinose and in some cases a uronic acid. By 1954 (17), improvements in knowledge about separation techniques allowed a group of workers in Pennsylvania to isolate from a fat- and pectin-free chlorite holocellulose a fraction, amounting to 60% of the hemicellulose, which contained xylose and glucose mainly, along with small amounts of arabinose, galactose and a uronic acid.

Although there is wide interest in grass chemistry from the agricultural point of view, most agricultural chemists have not been concerned with precise chemical information about the constitution of their natural materials and only isolated references have been found in the literature describing detailed chemical analyses. For example Philips and Davis (106) isolated, after purification and fractionation of a hemicellulose from lucerne hay, a product which contained only xylose and a trace of arabinose and a uronic acid which was neither galacturonic nor glucuronic but was thought to be a methyl ether of glucuronic acid.

Much more common are the analyses of the type described by Flanders (47) who studied the chlorite holocelluloses from various straws including lucerne and red clover. Lucerne yielded 85%

holocellulose, red clover 78%. From these the yields of hemicellulose were 34.1% and 42% respectively. He did not obtain pure fractions, but by using alkali of increasing concentration he showed that the ratio of moles of pentose per mole of uronic acid anhydride increased also. Some indications of the compositions of these hemicellulose fractions can be obtained from the following results.

Solvent	Lucerne Hay			Red Clover Hay		
	Uronic Anhydride	Pentose	Hexose	Uronic Anhydride	Pentose	Hexose
2.5% K_2CO_3	33.8	34.1	32.0	30.4	22.5	34.0
5% KOH	17.8	64.3	25.5	26.5	51.9	16.5
10% KOH	13.0	60.8	27.3	14.6	60.9	22.9
20% KOH	10.4	68.9	15.2	13.0	61.4	20.2

The fractions in both hays having the greatest uronic anhydride content are most readily removed by dilute alkali, as were the hexosans. With increasing strength of alkali, the pentose-rich fractions were dissolved. Practically all the hemicellulose will have been extracted by 20% KOH since (as stated earlier) 24% KOH is regarded as a solvent for all the hemicellulose in plant materials. Despite the general lack of interest in the individual polysaccharides of herbage, the galactomannans of lucerne and clover seeds have

been very closely studied and their structures described. The galactomannan may account for as much as 40% of the dry matter of the ungerminated seeds of these leguminous plants (6). In the seeds of "Provence" lucerne, there exists a water-soluble fraction which contains a galactomannan built up in the ratio of four units of galactose to five of mannose, while in a clover seed the ratio was found to be seven to nine. The monosaccharide units were thought to be combined through 1-4 or 1-6 linkages. Also from lucerne seeds, a galactomannan has been isolated (70) in which the galactose:mannose ratio was 2:1. Studies after methylation indicated a branched chain structure in which half the galactose residues were terminal groups. This sample was unusual in having a larger galactose content than mannose, since most galactomannans found contain a greater proportion of mannose (96).

Polygalacturonic acid occurs widely as pectin in the plant world. Polygalacturonides are only present to the extent of a few per cent of the dry matter in grasses but in leguminous plants they account for about 10% of the dry matter of the young plant. Glucuronic acid has been reported in the 4% sodium hydroxide-soluble hemicellulose fraction of the roots (19) of lucerne, and a methyl ether derivative in the aerial portions (106).

A report on work carried out on the relation between the lignin content, the cellulose content and the degree of

polymerisation of the cellulose in lucerne was presented by Francois to the European Grassland Conference in 1954 (51). The cellulose was prepared from different parts of the lucerne plant, nitrated and the degree of polymerisation (D.P.) deduced from the viscosity of the nitrates in butyl acetate. Some indication of the size of the cellulose molecule in lucerne can be obtained from the results.

Source of cellulose in lucerne	Cellulose		Lignin	D.P. of cellulose
	Crude*	Pure*		
Green leaves	17.8	13.25	10.00	306
Hay	21.7	18.20	11.25	700
	27.4	23.20	15.40	830
	31.2	25.09	13.00	840
	36.4	26.80	15.80	1050
Stems	45.0	32.40	16.50	1140
	45.2	37.10	17.35	1185

*[Crude cellulose - fibre ground, boiled in alcohol, ether, water (4 hrs.), then steeped in a 2% sodium solution for 4 hours.

Pure cellulose - further treatment of crude cellulose with sulphite and hyposulphite.]

The results indicated that during growth, the cellulose undergoes a modification which increases the length of chain and simultaneously the cellulose content increases.

The agricultural importance of herbage carbohydrate.

Pasture or fresh forage is the natural food of horses and of farm stock used for the production of milk and meat (139). The feeding value of any material is measured by the extent to which it satisfies the three main requirements of bulk, energy production and protein content. In general the first two of these are readily supplied as most farm foods are bulky and contain appreciable amounts of carbohydrates, which the animal can use for the production of energy. The supply of adequate amounts of protein presents a rather greater problem. The important sources of nitrogen in farm foods are young grass, the pulses and legumes.

Apart from empirical information from the amount of a feed consumed, the feeding value of any plant crop can be determined by observing the growth of an animal on a diet containing it alone or admixed with other substances. Some estimate of the potential feeding value of any material can often be obtained by chemical analysis, although Woodman et al. (148) have pointed out that predictions regarding the nutritive value are without justification if made on the basis of chemical composition alone, since an

animal may be unable to digest all the protein and carbohydrate in the feed.

The water-soluble carbohydrate is important from the point of view of its energy value for all animals but in addition to this, the hemicellulose can be utilised to a certain extent by ruminants (65,66). An increase in the amount of fibre in a plant results in a "woodiness" or "stemminess" and this is found to have a marked effect upon the digestibility of the carbohydrate. Fermentation of plant cellulose to fatty acids has been recognised as an additional energy source in ruminants.(38,92,132)

Watson in his book "Grassland and Grassland Products" (138) has traced the course of research carried out on herbage from the viewpoint of the agriculturalist, and the more important features of his review are given below in order to show how the work presented in this thesis may be correlated with earlier studies.

The earliest work on pure species of grass was carried out by Sinclair (119) in 1825. On the suggestion of Sir Humphrey Davy he used the amount of material extracted by hot water as the criterion upon which to base the nutritive value of the grass or plant material. In the middle of the nineteenth century Way (140) studied pure species of grasses (i.e. Gramineae) and what he called "artificial grasses" (the clovers) which were also used as farm feedingstuffs. His analyses, more detailed than Sinclair's pioneer work were for five different components and he found

differences between these two types of plants. The results, tabulated below, show the basic differences in composition characteristic of the two species - grasses and clovers, but accurate comparisons cannot be made since all his samples were not collected at the same time of the year.

		"natural"	"artificial"
Flesh-forming principles	i.e. Protein	10.98	19.03
Fat-producing principles	Fat	3.08	3.65
Heat-producing principles	N.F.E.	45.57	41.29
Woody-fibre	Crude fibre	33.55	26.23
Mineral matter (or Ash)	Ash	6.64	9.25

Composition and stage of growth.

Towards the end of last century, investigations were extended to studies of the changes in composition at various stages of growth, and Wilson (144) found that the stage of growth determined the nutritive value. He cut grass and clover plots at three stages of growth and concluded that "compared with the differences of composition at different stages of growth,

differences between the compositions of various grasses, cut at the same stage, are small". This similarity must not be assumed true for every grass because work by Waite (134) has shown that the growth characteristics of the grass must also be considered; for example a rapidly growing variety of grass will not have the same fructosan content as a slower growing variety. (This is discussed later in the thesis.)

In 1924, Fagan and his co-workers at Aberystwyth began a study of the composition of grasses and clovers. The results from the different grasses studied showed (like Wilson's work) that their compositions were similar if they were cut at the same stage of growth. (The same was also true of leguminous plants.) They found that with a rapidly growing variety of ryegrass (Italian) the changes which took place within weeks were marked (39).

Period of growth (weeks)	Material extracted by dry ether *	Crude Protein (N x 6.25)	True Protein (C.P.- Soluble N x 6.25)	Crude Fibre	N.F.E.	Ash
2	3.75	18.84	13.32	20.45	44.36	12.60
6	2.42	12.12	7.80	21.62	55.89	7.95
10	2.10	6.90	5.47	25.33	60.22	5.45

[All results as percentages of the dry matter.

*The ether extract represents mainly the fat content].

The results showed a fall in the percentage of all the constituents except the carbohydrate fractions i.e. fibre and N.F.E. Although all the nitrogen-free-extractives are not in fact carbohydrate molecules, the latter do form the greater portion of this fraction, and an increase in the N.F.E. value is assumed to reflect an increase in the amount of carbohydrate available to the animal.

The change in the protein content of the plant is the most marked. Both the soluble nitrogen (i.e. the fraction of the total nitrogen soluble in boiling water) and the true protein (i.e. [total nitrogen - soluble nitrogen] x 6.25) in the plant were found to decrease greatly with maturity.

Fagan has also stressed the importance of (i) yield of dry matter (ii) palatability and (iii) season of maximum productivity (40-44) in the evaluation of any plant crop.

(i) Yield of dry matter. The yield of dry matter obtained per acre of any field and the nutritive value of that dry matter are the two most important factors in the economics of any agricultural work. Unfortunately for the farmer, every hundred pounds weight of fresh herbage in his fields only contains some twenty pounds of dry matter and this high moisture content makes it necessary for the grass to be dried before being stored as hay. In 1927, it was found (3) that for both grass and lucerne, larger yields of hay per acre were obtained when the plants were

cut at a relatively mature stage. Cutting at the full flower stage was recommended by Griffith and Ramsay (54) and this was confirmed by Klapp (77) who showed that cutting between the twelfth and fourteenth weeks (approximately the same stage) gave the highest yields of lucerne.

(ii) Palatability. Some species of grassland clovers are grazed readily throughout the season. With grasses and leguminous plants, the stage of growth is thought to be the controlling factor, since the palatability of plants is dependent on the leafiness. As the plant matures, the proportion of stem increases and it becomes less attractive. Since clovers do not become as woody as lucerne or grasses, (the proportion of leaf is always high), they are readily consumed over the whole season. Regular grazing is beneficial in keeping the palatability of a pasture high, since the grass is not allowed to reach maturity and fresh young grass is continually being produced.

(iii) Season of maximum productivity. On account of the change in composition and yield of a plant crop over the summer season, there is naturally a stage at which it is most economical for harvesting. Different grasses, and even different strains of the same grass species, grow at different rates. Waite (134) has studied the chemical composition of various grasses with different growth characteristics and his results indicate a

probable inverse relationship between the carbohydrate reserve and the rate at which the grass grows.

Thus it is found that grasses and other plants may reach their optimum feeding value at different times of the year. This is of great importance to the farmer because using a mixture of seeds of various strains, his pastures have a high nutritive value over a longer period of the growing season.

The importance of leaf:stem ratio. It has been seen earlier that the amount of leaf in any plant plays an important part in the determination of its feeding value. Osvald (103) showed that there were differences between the chemical composition of the leaf and stem of grass at the hay stage. The differences were not so clear at this stage as in young grass, nevertheless the leaf seemed to be richer in protein and poorer in fibre than the stem. Fagan showed that these differences existed at each stage of growth and found that the leaf:stem ratio decreased as the plant matured. Therefore there can be a large overall change in the composition of a herbage crop due to these two factors.

Waite and Sastry (137) in a study of timothy grass, found that the leaf:stem ratio changed from 2.5 to 0.5 over the season. The changes in the protein composition of the leaf and the stem followed similar trends and these authors concluded that the value of the crop depended on its stage of growth. The protein

content fell, and the starch equivalent (a measure of the amount of carbohydrate available to the animal) increased with maturity. In the pattern of these changes, Kellner (74) and Fagan have shown that legumes and grasses are similar.

Weeks growth	Italian Ryegrass		Montgomery Red Clover	
	Crude Fibre	Crude Protein	Crude Fibre	Crude Protein
5	17.4	25.1	18.3	26.2
7	21.2	20.6	21.3	21.4
9	27.2	19.3	25.8	19.8
11	30.9	16.3	27.6	17.1

(All as percentages of the dry matter).

These results have been found to confirm the early work of Wilson (58,144).

Seasonal variations. The changes in composition of a plant crop do not proceed at the same rate throughout the whole season and it was noted (45) that compositional changes were greater during the period of flowering and just after, than in the early vegetative stage of growth. After the floral stage, clovers, and to a lesser extent grasses, showed a rapid increase in the amount of fibre.

Little detailed information is found in the literature on the seasonal variations in the carbohydrates of grasses and clovers. Cereal grasses have been shown to contain fructosan, with a maximum amount in the stem at the flowering stage (14,28,29,33,135) and the sucrose content of wheat plants during winter and early spring was found to be dependent on the temperature, although from March onwards there was a continuous fall.

Norman and his collaborators studied the seasonal variations of the carbohydrates of ryegrass (Western Wolths variety), and reported (100,102) an increase of xylan, cellulose and lignin with age. Fructosan passed through a peak and fell as maturity approached. The amount of fructosan in cocksfoot was shown (101) to be a maximum about three weeks before the time of full emergence of head, declining with further maturity, and Waite and Boyd (135) who analysed perennial ryegrass, timothy, cocksfoot and meadow fescue during two successive growth cycles, reported a peak in the amount of sucrose during May and June. The method of analysis used by the latter workers (described earlier) may include in the sucrose estimation, short chain fructosans which may account for an appreciable proportion of the alcohol soluble sugars (see the section on seasonal variations in perennial ryegrass).

In the timothy, cocksfoot and meadow fescue, the stem

fructosan contents showed two peaks but in the perennial ryegrass (S23 strain) only one was found. These changes have been correlated with the rate and stage of growth of the grasses (134,135) and similar relationships between carbohydrate content and growth have been suggested by Archbold (7) and McCarty (84).

Changes in the chemical composition of other forage grasses with maturity have been reported, but little information about variations in the amounts of separate chemical constituents can be obtained. The results indicate an increasing amount of cell wall materials (9,74,107,124,128) while other carbohydrates show a maximum at some stage in the season, usually about the stage of flowering.

Lucerne has not been studied in any greater detail, and, as for the grasses, little information about the carbohydrates can be gathered from the work which has been done. Early work on the problem was carried out by Swanson and Latshaw (128) who studied lucerne cut at the budding, one-tenth bloom, full bloom and seed formation stages. In each successive stage, the crude fibre and N.F.E. were found to increase while the protein content fell.

Thus it can be seen that the general trends which accompany the growth of grasses to maturity are found also in lucerne (3,16,54,126,146).

Diurnal variations. In addition to the changes occurring over the growing season, diurnal variations in the carbohydrate content

are found in plants. Henrici (67) observed that during the analysis of lucerne, the night and day samples did not give the same result. Approximate agreement was noted between two samples taken at twenty-four hour intervals except during changeable weather conditions. Higher N.F.E. values have been reported (36,149) for lucerne during daylight than at night and an increase in the amount of sucrose with daylight is found (34,91,93). Diurnal variations in the amount of water-soluble sugars in perennial ryegrass have been studied (91,135,150) and while the hexose monosaccharides were almost unchanged in amount, sucrose showed a maximum towards late afternoon (4.00 p.m. - 7.00 p.m.). The amount of fructosan present in the plant did not change as regularly as the sucrose, and it is likely that other factors controlled the quantity present.

The production of starch has been shown to depend on light, and starch formed during the hours of sunlight wholly or partially disappeared from mangold and potato leaves during the night (34). Miller (93) has found indications of a disappearance of polysaccharide material from the leaf of maize and of sorghum during the hours of darkness and it is possible that some of the carbohydrate may be moved to the roots as is suspected for lucerne (120).

Defoliation. The frequency with which a plant is cut or grazed is found to have a marked effect on its carbohydrate composition.

Waite and Boyd (136) found low water-soluble carbohydrate contents in S23 perennial ryegrass when it was cut every time it reached a height of 8-10 inches. This confirmed the observation that second and subsequent cuts have less soluble sugar than the first growth (125). No evidence has been found to suggest that the cellulose and hemicellulose of perennial ryegrass could be utilised for the recovery of the plant after defoliation, although the water-soluble sugars in both the roots and the stubble were depleted temporarily (127). This lasted until new foliage was able to photosynthesise enough carbohydrate for growth requirements.

Similarly with lucerne, clipping too frequently caused depletion of carbohydrate reserves, root (57) and above ground (94) development was affected and in extreme cases the death of the plant ensued.

From this summary of the work carried out on the carbohydrates in pasture crops, it can be seen that knowledge about the actual changes which take place in the growing plant cannot be obtained from these experiments using the older analytical methods. By using the newer techniques it is now possible without great effort to determine accurately the amounts of the various carbohydrate constituents present in plant tissues.

The work already carried out has shown that grasses and clovers have similar carbohydrate compositions with one very important exception. In the monocotyledons there is found a

fructosan which functions as a reserve polysaccharide; in the dicotyledons there is not. In many of the latter (e.g. lucerne) there is a starch present, and this is believed to perform the functions of the reserve. As examples of these two classes, perennial ryegrass and lucerne were selected for a detailed study using the chromatographic techniques.

Perennial Ryegrass (Lolium Perenne L.) is a valuable grazing and hay grass which is extensively sown in the British Isles (especially in lowland areas) and its use is world wide.

Lucerne (Medicago sativa L.) is also grown in many parts of the world. Known as Alfalfa in the Americas, its growth under conditions of careful cutting may be extended over a number of years, and it forms a valuable source of nitrogen and calcium.

The object of the work reported in this thesis was to study the various carbohydrate constituents in ryegrass and lucerne with particular reference to the changes occurring in them during the growth of the plant.

There is, in addition, a report on experiments carried out on the changes occurring in the roots of perennial ryegrass and the inter-relation of the carbohydrate contents of the above- and below-ground portions of that plant.

The conclusions reached after examination of the analytical results, are that the changes found in the non-cell-wall carbohydrates are due to the demands on the carbohydrate being photosynthesised by the plant for flower and seed development or the production of new tissue. Any carbohydrate which is synthesised in excess of the plant's immediate requirements is built up into the "reserve" polysaccharide. This balance between synthesised and "reserve" carbohydrate also appears to be related with the carbohydrates in the roots - some of the carbohydrate in the above-ground portions being transferred to the roots if not required. As with the reserve polysaccharide in the leaf and stem, it is not possible to decide whether such carbohydrate is "lost" to the plant for energy purposes or whether it is being only temporarily stored against future demands.

The changes found in the cell-wall polysaccharides have confirmed the general trends previously noted, but it has been possible to show that variations in the proportions of the constituent sugars do occur.

PART 1. EXPERIMENTAL

Seasonal variations of the carbohydrate constituents in Perennial Ryegrass and Lucerne.

Experimental.

Preparation of samples for analysis.

The grass and lucerne were grown in small plots (approx. 1/20 acre) which were divided into squares and for each sample taken, the grass or lucerne from five or six random squares was cut 1-1½ inches above ground level. The cut material was mixed thoroughly in the field and a representative sample taken for analysis. For grass samples, the leaf was removed at the ligule and the stem therefore included the culm and the leaf sheath. The separation of leaf and stem of lucerne was more distinct - only the leaf material being included in that sample. Where flowers or seeds were also present on the plant, these were separated from the stems.

Samples for carbohydrate analysis and moisture determination (about 30 gm. fresh weight for each) were weighed out. The former samples were placed in hot alcohol (50-60°C.) brought to the field in thermos jugs, and the latter were transported back to the laboratory and placed in an oven at 80°C. within 1 hour of cutting. The time required for cutting and dissection was generally 25-30 minutes.

Free sugar extraction.

The alcohol used for the deactivation of the enzymes was decanted from the plant material and the residue was rapidly disintegrated using a top-drive macerator. The sample was transferred quantitatively to a Soxhlet thimble (27 x 250 mm.) and extracted with 80% (v/v) ethanol (vapour = 90% ethanol) for 6-8 hours.

All the alcohol solutions were combined and the volume noted. An aliquot (equivalent to ca. 1 gm. dry grass or to 2 gm. dry lucerne) was taken and a sample of xylose (about 20 mg.) was added as a reference sugar. An equal volume of water was added to the aliquot and the alcohol evaporated at 35°C. under reduced pressure. Further portions of water were added, if required, until all the alcohol was removed. The volume was reduced to 100-150 cc.

Clarification of plant extracts.

Clarification of plant extracts was carried out by coprecipitation with cadmium hydroxide and barium sulphate (82). A saturated aqueous solution of barium hydroxide was prepared. A small amount of water was added and the solution stored in a bottle protected from carbon dioxide with a soda lime trap.

Cadmium sulphate solution (4.6 gm. in 100 cc., ca. 0.36N) was also prepared.

Before use 10 cc. of cadmium sulphate solution were titrated against the barium hydroxide solution to determine the equimolecular volume. The plant extract (after removal of alcohol) was heated to 95° on a boiling water bath. The mixture was maintained at this temperature for 2 minutes after the simultaneous addition of cadmium sulphate (10 cc.) and barium hydroxide solution (equimolecular volume). The solution was rapidly cooled in running water and filtered through a pad of "supercel" (a filter aid) on a Buchner funnel.

Deionisation of sugar solutions.

Deionisation after clarification in this work has been by electrodialysis. The apparatus used was similar to that described by Macpherson (90). It consists of a perspex cell which was divided into three compartments by two membranes. The cathode membrane was of parchment and the anode, of cloth impregnated with gelatin (10%) hardened in formaldehyde (10%) for six hours. The solution to be deionised was placed in the central compartment while the two electrode compartments were filled to this same level with water. Cooling coils in the electrode compartments and efficient stirring of the central compartment containing the solution were essential. The voltage used was ca. 130v. (d.c.) and the time required for complete deionisation was 20-25 minutes.

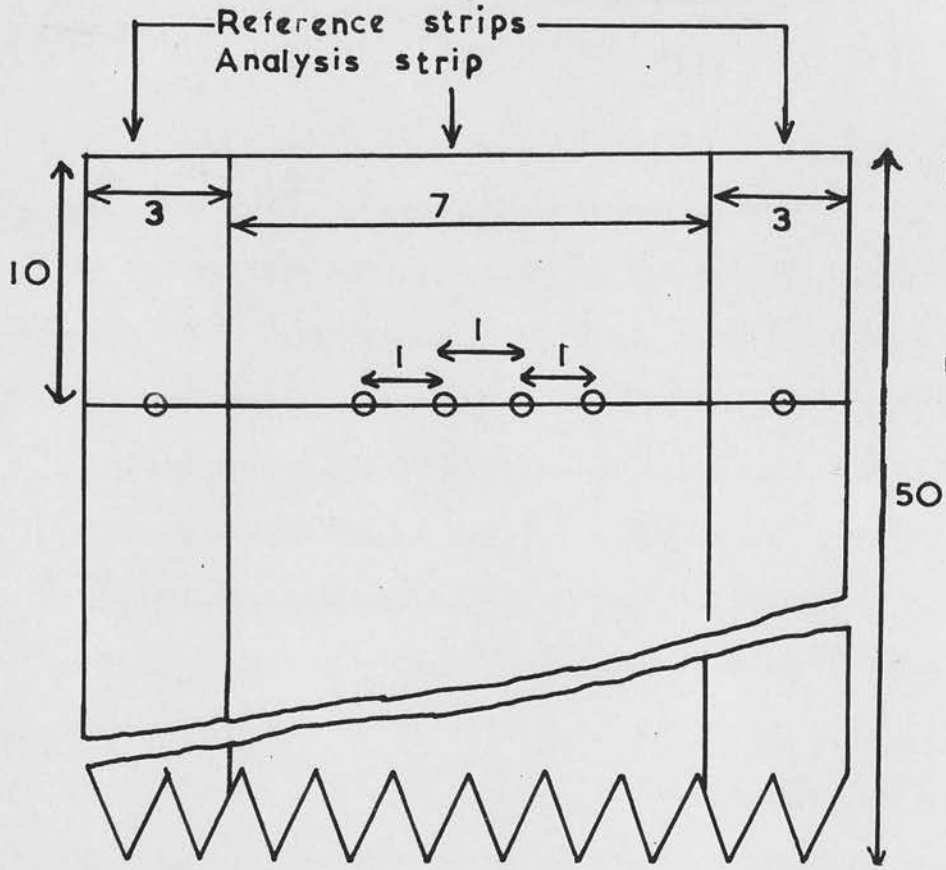
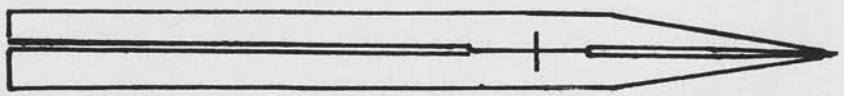


DIAGRAM 1.

measurements in centimetres



Scratch

DIAGRAM 2.

During the last stages of the experimental work, in place of the parchment and cloth - gelatin membranes, sheets of ion-exchange resins were used as membranes. These had been shown (5) to permit rapid and efficient deionisation.

Paper chromatographic separation of carbohydrates.

Whatman No.1 chromatography paper was used in strips (13 cm. x 50 cm.). The starting line was drawn 10 cm. from the top of the paper and the lower end was serrated to allow the solvent to drip evenly from the paper. Duplicate chromatograms for quantitative analyses were drawn out as shown (diagram 1), and equal volumes of the solution to be analysed were applied to the analysis strip from a micro-pipette (diagram 2). This pipette (approximate volume 0.03 cc.) was made from thick glass tubing (2 mm. internal diameter). One end of the tube was drawn out to a fine point and a constriction was formed 2 cm. from the tip. The pipette was always filled above the level of a scratch opposite the constriction, the amount of solution in the pipette being reduced to this volume exactly, by removing the excess from the pipette by touching the tip with a filter paper.

The following solvents were used for the separation of the sugar mixtures -

Solvent (i) ethyl acetate, acetic acid, water (3-1-3 v/v) (72) which gave a clear separation of sucrose, glucose, fructose, xylose and ribose,

Solvent (ii) n-butanol, benzene, pyridine, water (5-1-3-3 v/v)(141) which separated glucose, galactose, arabinose and xylose,

Solvent (iii) ethyl acetate, pyridine, water (25-10-35 v/v)(86) which separated glucose, galactose and mannose.

Other solvents were used in special experiments and these are reported in the relevant experimental section.

For the detection of sugars on paper chromatograms, various spray reagents have been employed. These are shown in the following table.

Reagent	Ref.	Spray	Heating conditions	Sugars detected	Colour
Aniline oxalate	104	Satd. aq. solution	4-5 minutes at 160°C.	Hexoses Pentoses Rhamnose Uronic acids	Brown Pink Yellow-brown Brown
Urea oxalate		Satd. aq. solution	5-10 minutes at 100°C. 10-15 minutes at 160°C.	Fructose or fructose in e.g. sucrose Hexoses) Pentoses)	Black Faint grey-brown
Silver nitrate	130	Dip paper in $AgNO_3$ (in acetone) Spray NaOH in ethanol	None	Reducing sugars and polyhydroxy compounds	Dark-brown stain
Naphtho-resorcinol	50	10 cc. 1% solution in EtOH + 90 cc. 2N HCl.	10 minutes 85-90°C.	Fructose Hexoses Pentoses Uronic Acid	Brown Grey Blue-grey Blue

When a sugar solution was to be analysed quantitatively, the contents of the pipette were distributed evenly on the four spots on the centre strip by spotting each one several times, drying the spots with a warm air blast from a hair drier after each application. The same volume of solution was spotted onto the reference strips, distributing it evenly between the four spots, one on each of the four reference strips of the duplicate chromatograms.

The papers were irrigated for about 40 hours in air-tight glass chromatography tanks. These were contained in a box in which the air (thermostatically controlled at 22.5±1°C.) was circulated. The organic phase of the solvent system was used for the separation of the sugars by descending solvent chromatography and a dish containing the aqueous phase was placed in the tank to maintain the atmosphere in equilibrium. The papers were dried in air at room temperature for 30-45 minutes; the side strips were cut off and sprayed with a suitable reagent. These showed the positions of the sugars on the analysis strip. The sugar zones were cut out and the sugars extracted with hot water by the method of Flood, Hirst and Jones (48). The paper strip containing the sugar was suspended from a hook attached to a reflux condenser above a tube fitted with a ground-glass joint. The tube contained 5.5 cc. water which was boiled and the condensate ran over the hook and down the strip dissolving the

sugar. One hour under reflux was required for quantitative extraction of monosaccharides from Whatman No.1 paper. In order to extract oligosaccharides it was necessary to continue refluxing for a further 30 minutes.

It was found convenient to extract and hydrolyse sucrose simultaneously. The paper strip was hung above a solution of oxalic acid (5.5 cc., 1% w/v). The sucrose was washed off the paper by the condensate and hydrolysed by the boiling oxalic acid. It was necessary to boil the oxalic acid solution for 1¼ hours to obtain complete extraction and hydrolysis of the sucrose.

Cold water extraction of sugars from chromatograms.

Distilled water was run from a capillary tipped dropping tube down the paper strip held between two glass rods (81). The eluate (5 cc. was sufficient to wash all the monosaccharide off the paper) was collected in a test-tube. The method was not used for sucrose or oligosaccharides.

Comparison of hot and cold water methods for sugar extraction from paper strips.

In order to compare the analytical results from the two methods, the following sugar mixtures (20 mg. approx. of each sugar dissolved in ½ cc. water) were prepared and analysed.

(a) galactose, arabinose and ribose (separated in solvent ii).
(b) glucose and fructose (solvent i).
(c) mannose and xylose (solvent i).

The groupings were chosen to allow complete separation of sugars on the chromatograms.

Four replicate paper chromatograms were prepared using small portions (ca. 0.03 cc.) of these three solutions. One pair of each set of papers containing the three mixtures was eluted with hot, and the other pair with cold water. The sugars in the eluates were estimated by the Somogyi method (122) details of which are given on page 40 . The average results of the duplicate sugar analyses are presented in the following table. The differences (D) in the amounts of sugar extracted are expressed as percentages of the sugar removed by cold water.

	Sugar	Hot (mg.)	Cold (mg.)	D	% Difference
Mixture (i)	Arabinose	0.828	0.834	0.006	- 0.7
	Galactose	0.642	0.661	0.019	- 2.9
	Ribose	0.789	0.770	0.019	+ 2.5
Mixture (ii)	Fructose	0.549	0.575	0.026	- 4.5
	Glucose	0.690	0.683	0.007	+ 1.0
Mixture (iii)	Mannose	0.427	0.415	0.012	+ 2.9
	Xylose	0.618	0.598	0.020	+ 3.3

The results showed little difference between the two methods and confirmed the recoveries found by earlier workers (49). The hot water technique being the more convenient, it was decided to use it in all cases for sugar extraction from chromatograms.

Sugar estimations.

The original Somogyi reagent (118) has been modified three times, (121,122,123), the 1945 reagent being the one chosen for use in this work.

The reactions upon which the method is based are

(a) cupric ion in alkaline solution reduced by the sugar to cuprous oxide,

(b) cuprous oxide oxidised by iodate back to the cupric state, and

(c) the excess iodate measured by titration, with standard thiosulphate, of the iodine liberated on acidification.

The following solutions were prepared and the Somogyi reagent made up shortly before use.

A) alkaline copper sulphate solution.

B) potassium iodate - 0.25N.

C) potassium iodide - 2.5%(w/v).

D) sulphuric acid - 2N.

E) standard sodium thiosulphate (exactly 0.005N).

The amount of potassium iodate (B) present in the Somogyi reagent was regulated by the amount of sugar expected to be present in the 5 cc. of solution used in the determination. Details of the preparation of the Somogyi reagent are given in the table.

	mg.sugar in 5 cc.	Volume of KIO_3(ml.)	Volume of water(ml.)	Volume of solution A(ml.)
4 ml.reagent	0-1	4	6	90
6 ml.reagent	1-2	6	4	90
10 ml.reagent	2-3	10	0	90

To 5 cc. of the sugar solution in a test-tube fitted with a ground glass joint containing for example 2.5 mg. of reducing sugar, 5 cc. of the"10 ml. reagent" were added and the tube shaken. A glass bulb was placed in the neck of the tube to prevent excessive evaporation and the mixture was heated on a boiling water bath for 30 minutes exactly. The tube and its contents were cooled for 10-15 minutes in running water. Potassium iodide (2 ml.) was added carefully down the side of the tube, 1.5 cc. of acid (D) were added quickly from a pipette of wide delivery bore and the tube stoppered immediately and shaken. [For the 6 ml. and 4 ml. reagents the volume of KI was 1.5 ml. and 1 ml. resp.]. The liberated iodine was titrated with sodium thiosulphate (E) using 0.5% starch solution as indicator.

A blank determination using 5 ml. of distilled water was carried out simultaneously or if sugars from paper chromatograms were being estimated, the eluate from a blank strip of the irrigated paper of similar size was used.

Since the reduction in this reaction is not 100% of the theoretical, the reducing power of the different monosaccharides varies slightly. It was necessary therefore to determine the conversion factor (the "Somogyi factor") for each sugar. These factors were calculated from the volume of thiosulphate (E) equivalent to a known weight of sugar,

(B-T) x factor = weight of sugar in 5cc. of standard solution, where

B = volume of thiosulphate for the blank determination, and

T = volume of thiosulphate for the sugar determination.

The factor thus represents that weight of sugar (in mg.) which is equivalent to 1 ml. of 0.005N sodium thiosulphate.

The Somogyi factors were checked for each batch of stock solution (A) prepared. The slight variations in these factors found during the course of this work are recorded in the table

Arabinose	0.147	0.145	0.143	0.143	0.148
Fructose	0.140	0.137	0.135	0.135	0.138
Galactose	0.162	0.162	0.162	0.158	0.154
Glucose	0.130	0.129	0.130	0.130	0.127
Mannose	0.139	0.139	0.139	0.139	0.139
Ribose	0.199	0.186	0.188	0.186	0.186
Xylose	0.137	0.128	0.129	0.132	0.136

The results showed good agreement between batches.

Sucrose estimation.

After hydrolysis of a standard solution of sucrose with oxalic acid, the total reducing power of the hydrolysate [neutralised with NaOH or KOH (4N added dropwise)] gave a factor in the same manner as the monosaccharides

Sucrose	0.134	0.132	0.132	0.129	0.130

Oligosaccharide estimation.

An aliquot of the paper eluate of the oligosaccharides was made 0.5N w.r.t. H_2SO_4 and hydrolysed for 4 hours on a boiling water bath under reflux. Fructose was determined in another aliquot by the colorimetric method described for fructosan, and by applying a correction factor for the decomposition of fructose during hydrolysis [28% under these conditions (152)], the total weight of oligosaccharides can be calculated (see page 73).

Fructosan estimation.

The fructosan in the 80% alcohol-extracted perennial ryegrass was removed by cold water extraction (page 46) and estimated by the colour reaction with resorcinol, glycerol and HCl(10). This was a modification of a method developed by Roe (113). The method could be used for single fructose molecules or for the fructose contained in oligosaccharides and fructosan.

The reagents required were,

glycerol-HCl - (130 gm. glycerol B.P., 50 cc. water, 100 cc. conc. HCl containing 45 mg. $CuSO_4.5H_2O$ per litre).

resorcinol - 0.45%(w/v) resorcinol in water.

The sugar solution (2 cc.) containing 0.04-0.14 mg. fructose was heated with 5 cc. of the glycerol-HCl reagent and 1 cc. resorcinol, for 12 minutes exactly on a boiling water bath. After cooling (10-15 minutes in running water), the intensity of the colour was measured in a Spekker photoelectric absorptiometer, using violet filter - 601 (maximum transmission at 630 mμ wavelength). The amount of fructose was found from comparison of the reading with a previously determined standard curve.

Acid hydrolysis of plant polysaccharides.

In the analysis of the plant polysaccharides, the scheme has involved their hydrolysis with sulphuric acid [(58) and p. 73]. After addition of ribose as a reference sugar, the hydrolysates were neutralised with barium carbonate (A.R.) at room temperature. The insoluble salts were filtered off using a pad of supercel and the solution was evaporated to a small volume (ca. ½ cc.) under reduced pressure at 30°C. Before spotting the solution onto a paper chromatogram, a second filtration of the solution through a small filter paper was sometimes necessary to separate barium salts which were insoluble in this small volume of liquid. The

monosaccharides in the hydrolysates were separated by chromatography and the sugars eluted and estimated (as (58) and page 73).

Determination of the moisture content of herbage.

The fresh sample was oven-dried at 80°C. to constant weight (6-10 hrs.). No correction has been applied for loss of volatile acids or other organic material.

Determination of the crude protein, ash, ether-soluble material and uronic anhydride contents of dried herbage samples.

Crude protein.- The total nitrogen content of the oven-dried, milled grass or lucerne was determined by the micro-Kjeldahl method (26). The crude protein content is the total nitrogen content x 6.25.

Ash.- Ash determinations were carried out in silica crucibles on oven-dried samples of the grass or lucerne. The plant material (1 gm.) was ignited to constant weight over a Meker burner and the resulting ash weighed after cooling 15 minutes in a desiccator.

Ether-soluble material.- The oven-dried milled material (1 gm.) was shaken for 6 hours at room temperature with anhydrous di-ethyl ether (100 cc.). The residue was filtered off and washed with dry ether (3 x 100 cc. portions). The ether extract was evaporated at approx. 20°C. under reduced pressure. The extracted material was dried over P_2O_5 (in vacuo) at room temperature, and weighed.

Uronic anhydride.- The method used was that of McCready, Svenson and Maclay (85). The oven-dried milled plant material (0.5 gm.) was heated with 19% HCl (30 cc.) at 145°C. for 1½ hours. The CO_2 evolved was absorbed in a known quantity of carbonate-free sodium hydroxide. Hydrochloric acid fumes from the reaction flask were prevented from reaching the alkali by a granulated tin and zinc dust trap and furfural derivatives (from pentosans) were removed in an aniline bubbler. The results, calculated from the CO_2 evolved, were expressed as percentages of uronic anhydride.

Analysis of samples of perennial ryegrass - 1953.

In the study of the seasonal variations of the carbohydrates in perennial ryegrass, the analytical methods used were developed by Wylam (152) and Harwood (58).

In collaboration with Dr. C.B. Wylam, the author carried out the analyses of the second growth of the ryegrass. The scheme for analysis comprised the extraction of the glucose, fructose, sucrose and oligosaccharides with 80% ethanol from a macerated sample in a Soxhlet apparatus for ca. 8 hours (p. 33). The solution was clarified and deionised (pp. 33, 34) and the sugars estimated by the Somogyi method after chromatographic separation using solvent (i).

The fructosan was removed by shaking the residue from the alcohol extraction overnight with water (500 cc.) The residue was

filtered off and the extraction repeated for a second night. The residue was again filtered and washed with cold water. The fructosan in the combined extracts and washings was estimated by the colorimetric method.

The polysaccharides were analysed by Harwood's method. Since there was no information about the constitution of the actual polysaccharides present, the results were expressed as percentages of the anhydro-sugars in the dry weight of the original sample i.e. as polyarabinose, polyxylose etc. in the plant.

Analysis of samples of perennial ryegrass - 1955.

The method of cultivation was sand culture, and a mixture of sand was chosen [2 parts by weight of Leighton Buzzard sand (coarse) to 1 part Lochaline sand (fine)]. The nutrient solution (recommended by Mr. Eadie of the East of Scotland College of Agriculture who had used it for pot cultures of ryegrass) was made up as follows.

Four stock solutions and a stock solution of trace elements were prepared.

A -	Calcium nitrate	- 72 gm. per litre
B -	Sodium di-hydrogen phosphate	- 56.5 gm. per litre
C -	Potassium sulphate	- 40 gm. per litre
D -	Magnesium sulphate	- 26 gm. per litre

E - Trace elements

Copper sulphate	- 1 gm.	Dissolved in 500 cc. water
Zinc sulphate	- 0.75 gm.	
Ferrous sulphate	- 0.75 gm.	
Borax	- 0.5 gm.	
Manganese sulphate	- 0.5 gm.	

For application, 1 cc. of A, B, C and D were mixed with 96 cc. water and to every 350 cc. of this dilute solution applied to the sand, 1 cc. of the trace element solution was added.

The sand was placed in orange boxes (to a depth 9-10 inches) which were in a greenhouse electrically heated so that the temperature was not below about 60°F. (15°C.) [see photograph]. The grass (S24 perennial ryegrass) was sown in rows during the first week of April 1955. The amount of nutrient solution applied (twice weekly) was 1 litre per section of an orange box i.e. 2 litres per box. The approximate volume of the sand per section was just more than 1 cubic foot ($14\frac{1}{2}$ x $13\frac{1}{2}$ x $9\frac{1}{2}$ cubic inches). In addition to the twice weekly application of nutrient solution, it was necessary to water the grass daily to prevent the sand from becoming too dry.

At the beginning of August, the boxes were placed outside the greenhouse to avoid the very high temperatures found inside the house.

When a sample was taken, one side of a section of a box was completely removed. Starting from the foot of the box, as much

sand as possible was gently shaken off the roots by hand; the three rows in the section being gathered one after the other. The plants, to which much sand still adhered, were transported back to the laboratory.

The roots were cut from the stems with scissors. (The roots begin at a definite point, so no difficulty was experienced about selecting the point of separation). The root material was then agitated gently (by hand) in the washing medium (acetone was finally selected - p. 50), to remove as much of the adhering sand as possible. This washing was repeated twice with fresh portions of acetone and the roots were then placed in a glass dish under a current of cool air to remove the acetone. The residue was weighed and representative sub-samples taken for carbohydrate analysis and for the determination of the dry ash-free weight, the former sample being immersed in boiling alcohol. The time since cutting was 25-30 minutes. The aerial portion of the plant was weighed and samples of the leaf plus stem taken for moisture and carbohydrate analyses, the latter being immersed in boiling alcohol.

Washing media.

The following liquids were tried.

i) chloroform - it was found very difficult to remove the adhering sand particles in this liquid.

ii) acetone - this was very suitable. It allowed quick removal of

the sand and the excess liquid could be blown off the roots in a current of cold air in a few minutes. The solubility of the sugars in acetone was a disadvantage.

iii) ethyl methyl ketone - this liquid did not permit such quick separation of the sand as acetone, and being less volatile, it was more difficult to remove afterwards.

iv) water - the sand separated fairly readily but the liquid could not be removed as conveniently as acetone.

A comparative experiment using acetone and ethyl methyl ketone was carried out. Two samples (approx. equal in size) were weighed out. The sand was washed off in each liquid for the same length of time using similar techniques. The excess solvent was evaporated in a cold air blast for 4 minutes; the samples were weighed and oven-dried (80°C) overnight. Ash determinations (by ignition with a Meker burner to constant weight) were carried out on the dry samples.

Washing liquid	"Moisture" (%)	Ash (% dry wt.)
Acetone	41%	26%
Ethyl methyl ketone	55%	61%

These results showed that acetone allowed much more ready removal of the sand and also left a "drier" sample from which the sub-samples were weighed out.

Ash content of a sample exhaustively washed with water.

A sample (20 gm.) of roots contaminated with sand was washed thoroughly with running water over a wire gauze. The sand was removed as completely as possible and the sample was oven-dried at 80°C. overnight. A portion (0.3076 gm.) of the dried root was carefully ignited in a silica crucible over a Meker burner. The ash was weighed after cooling in a desiccator. The weight of ash was 47.1 mg. i.e. 15.34% of the dry root.

Method for the analysis of root carbohydrates.

The analysis sample was separated from the alcohol used to deactivate the enzymes, and macerated in alcohol. The roots broke down fairly readily and the macerated sample was transferred quantitatively to a Soxhlet thimble.

The mono-, di- and oligo-saccharides were extracted with 80% ethanol during a 10 hour period (the extracting alcohol was changed after 6 and 8 hours). The three extracts A (0-6 hours), B (6-8 hours) and C (8-10 hours) were examined for sugar contents. An aliquot of the acetone washings equivalent to the proportion of the washed sample taken for carbohydrate analysis was analysed for the sugar extracted during washing.

A) - An aliquot (200 cc.) from the solution (1600 cc.) was taken. Xylose was shown to be absent and 22.2 mg. of that sugar was added as a reference quantity. The solution was analysed using the method described for ryegrass (p. 46).

B) - Xylose (5.4 mg.) was added to the extract and the sugars present were estimated. [Sucrose and oligosaccharides were combined before hydrolysis with 0.5 N H_2SO_4 (page 43). The value was corrected for fructose loss as for the ryegrass leaf and stem oligosaccharides.

C) - No traces of sugar were detected in this extract.

D) - The acetone washings were analysed by the method described for (A) above.

The results (expressed as mg. sugar found in each extract) are shown in the following table.

	Glucose	Fructose	Sucrose	Oligosacchs. (Somogyi factor 0.135)
A - (0-6 hr.)	40.68	30.52	123.62	29.05
B - (6-8 hr.)	trace	nil	0.59	
D - (Acetone)	5.24	5.97	2.91	
Total wt.	45.92	36.49	156.17	
% Dry ash-free weight of roots	0.53	0.42	1.79	

The proportion of the sugar found in the acetone washings was 5.9%.

Adsorption of sugars on the sand.

The sand from the acetone washings was separated and washed

thoroughly with hot water (10 portions; each 50 cc.). The solution was evaporated to small volume (at 30°C) and spotted onto a chromatogram irrigated (solvent i) for 40 hours. No traces of sugars could be detected.

Cold water extraction.

A sample of ryegrass roots which had been extracted with 80% ethanol for 8 hours was shaken with cold water (as p. 46). The volume was reduced (30°C., reduced pressure) and the fructosan was estimated by the colorimetric method. It accounted for 0.21% of the dry ash-free weight of the roots. Examination of the aqueous extract on a chromatogram (irrigated with solvent ii) after hydrolysis (1N H_2SO_4 - 5 hrs. boiling under reflux) showed that fructose was the main sugar present and only traces of glucose and xylose could be seen by examination of the chromatogram (sprayed with aniline oxalate) in ultra violet light.

Normal sulphuric acid treatment.

A sample (2 gm.) of ryegrass roots which had been extracted with 80% ethanol and cold water (as above) was placed in a flask and extracted on a boiling water bath with H_2SO_4 (1N; 60 cc.) for 15 minutes. The residue was filtered off and treated with a fresh portion of acid (1N; 30 c.c.) (previously heated on the water bath). The procedure was repeated until six 15-minute extractions had been carried out on the sample. The 6 separate

extracts were boiled under reflux for 4 hours. The hydrolysates were neutralised ($BaCO_3$) and examined qualitatively for the monosaccharides present by paper chromatography (using solvent ii).

The chromatographic results are shown in the following table.

Extract No.	Heating time (mins.)	Monosaccharides present in the hydrolysate
1	0-15	Large amounts of galactose, arabinose, xylose Small amount of glucose
2	15-30	Large amount of xylose Small amounts of galactose, arabinose, glucose
3	30-45	Large amount of xylose Small amounts of galactose, arabinose, glucose
4	45-60	Small amounts of glucose, xylose Traces of arabinose No galactose detected
5	60-75	Small amounts of glucose, xylose Arabinose just detected in trace amount No galactose detected
6	75-90	Small amounts of glucose, xylose No galactose or arabinose detected

It appeared that 75 minutes were required for removal of all the arabinose residues. The galactose-containing material was dissolved in 45 minutes. Prolonging the extraction led to greater removal of xylose and glucose.

Hydrolysis of the cellulose with 72% sulphuric acid.

A sample of ryegrass root was extracted with 80% ethanol (8 hours), cold water (twice for 16 hours) and sulphuric acid (1N; 1¼ hours on a boiling water bath). The residue was washed and dried at 80°C. overnight.

Two samples were weighed out (I 0.3469 gm. II 0.3424 gm.) and each was hydrolysed with 5.25 cc. 72% (w/w) H_2SO_4 for 4 hours at room temperature. The two solutions were diluted with water (125 cc.) and boiled under reflux for 2 hours (I) and 3 hours (II). Ribose was added (I 21.0 mg.; II 17.1 mg.) to aliquots of the hydrolysates (I 60 cc. from 230 cc.; II 60 cc. from 220 cc.), and the solutions were quantitatively analysed after chromatography. The ash content of the material was 3.4% and the crude protein 8.0%.

	I	II
Weight of glucose on paper (mg.)	0.468	0.764
Weight of xylose on paper (mg.)	0.103	0.144
Weight of ribose on paper (mg.)	0.225	0.262
S glucose (gm.) (see p. 75)	0.1506	0.1645
S xylose (gm.) (see p. 75)	0.0324	0.0303
Weight of ash-free lignin	0.0835	0.0854
P - recovery of carbohydrate (see p. 75)	81.8%	89.4%

The conditions selected for the analysis of the cellulose residue were 4 hours treatment at room temperature with 72% H_2SO_4 followed by 3 hours boiling under reflux as a 1N acid solution.

Analysis of the leaf and stem.

The above ground portion of the plant was free from much sand contamination and was analysed by the method described for the leaf and stem samples in the seasonal analyses 1953 (p. 46). From the weight of the sample and the number of tillers it was possible to calculate the average weight per tiller.

Growing points.

By carefully removing the leaves and leaf sheaths of the grass, the minute growing point could be obtained and examined under a low power microscope. The change from the vegetative to the floral state can be observed by study of this growing point. In the vegetative state, it had the form of a smooth-sided cone. In ryegrass, the silhouette of the growing point, as viewed under the microscope, becomes uneven as if tubular rings were being formed round the cone when it changes to the floral state. This gradually develops and emerges as the flower head.

Preliminary examination of lucerne.

The conditions for the estimation of the sugars in ryegrass were used in a preliminary experiment to determine the nature of the carbohydrates found in lucerne.

The 80% ethanol extract (8 hours in a Soxhlet apparatus) was shown, after paper chromatographic examination, to remove the same

sugars as from ryegrass viz. glucose, fructose, sucrose and a small amount of oligosaccharides.

The insoluble residue was shaken with cold water as for the fructosan extraction. No fructose was detected in the extract (colorimetric determination). After hydrolysis of the aqueous extract as a sulphuric acid solution (1N; 4 hours boiling under reflux) glucose, galactose, arabinose and xylose were detected on chromatograms of the hydrolysate (irrigated with solvent ii).

Treatment of the cold water extracted residue with 1N H_2SO_4 (1 hour on a boiling water bath) followed by hydrolysis (1N H_2SO_4; 4 hours boiling under reflux) of the extract gave galactose, glucose, arabinose and xylose (chromatogram run in solvent iii). There was also present (detected on a chromatogram run in solvent i) a very small amount of a sugar running at the same rate as rhamnose and a uronic acid (detected by naphtho-resorcinol spray).

The 72% H_2SO_4 treatment of the residue and final hydrolysis as a 1N acid solution (4 hours at room temperature: 3 hours boiling under reflux after dilution) hydrolysed the cellulose and remaining xylan in addition to liberating some mannose and residual galactose (detected on a chromatogram irrigated with solvent iii).

Development of the method for analysis of the carbohydrates in lucerne.

Extraction of the mono-, di- and oligosaccharides.

A sample of oven-dried milled lucerne (5 gm.) was weighed into

a Soxhlet thimble. Ethanol (80% (v/v); 400 cc.) was placed in the receiving flask and the material extracted for 4 hours. The alcohol in the receiver was changed (400 cc.) and the extraction continued for a further 2 hours. The alcohol was again changed (400 cc.) and the lucerne extracted for another 2 hours. The alcohol was evaporated after addition of an equal volume of water to the extracts and the solutions were concentrated (30°C.,reduced pressure) and made to a known volume.

Extract 1 - (0-4 hours) - volume 160 cc.

i) An aliquot (50 cc.) was clarified and deionised. The solution was evaporated (at 30°C., reduced pressure) to approx. 0.5 cc. A chromatogram was spotted with the solution three or four times using a fine capillary tube and irrigated with solvent i. The paper was sprayed with aniline oxalate and glucose, fructose, sucrose and a small amount of oligosaccharides were detected. There was no trace of xylose.

ii) Xylose (22.0 mg.) was added to a fresh aliquot (50 cc.). The solution was treated as above and spotted (ca. 0.03 cc. from ½ cc.) onto each of duplicate chromatograms for quantitative analysis (solvent i). The results (expressed as percentages of the dry weight of the original lucerne sample) were glucose 0.21%, fructose 0.26%, sucrose 4.75%, oligosaccharides 0.67%, i.e. a total of 5.89% or 295 mg. sugar soluble in 80% ethanol.

Extract 2 - (4-6 hours) - volume 60 cc.

An aliquot (20 cc.) was evaporated (30°C., reduced pressure) to 5 cc. The solution was acidified (½ cc.; 6N H_2SO_4) and hydrolysed for 4 hours on a boiling water bath. The total reducing value (estimated by Somogyi reagent) was 1.19 ml. of 0.005N sodium thiosulphate i.e. 3.57 ml. for the whole extract. This was equivalent to 0.46 mg. sugar (expressed as glucose).

Extract 3 - (6-8 hours) - volume 5 cc.

After hydrolysis, the total reducing value was estimated (as in 2 above). It was found to be 0.61 ml. thiosulphate or 0.08 mg. sugar (as glucose).

Conditions for the acid hydrolyses of lucerne polysaccharides.

A sample (100 gm.) of oven-dried milled lucerne was extracted with 80% ethanol for 8 hours in a Soxhlet apparatus. The residue was oven-dried (80°C. overnight) and used in the following experiments 1-4.

1) Normal sulphuric acid extraction.

Comparative experiments were carried out to determine the action of 1N sulphuric acid.

Five samples (approx. 5 gm. each) of the lucerne were weighed into flasks and extracted on a boiling water bath with the acid

(30 cc. per gm. of sample). After 30, 45, 60, 90 and 120 minute periods, one flask was removed and the residue filtered and washed thoroughly with water.

Aliquots (equivalent to about 0.3 gm. of the original residue) were made 1N w.r.t. H_2SO_4 and boiled under reflux (4 hours). The solutions were neutralised ($BaCO_3$) after addition of a reference sugar (20 mg. approx. of ribose) and the sugars present were separated on quantitative paper chromatograms (solvent iii). In addition to galactose, glucose, arabinose and xylose, there was a very small amount of a sugar which gave a yellow-brown stain with aniline oxalate and which travelled at the same rate as rhamnose. This was not estimated. No detectable amounts of unhydrolysed oligosaccharides were present on the chromatograms after the four hour hydrolysis.

Sample		A	B	C	D	E
Time in contact with H_2SO_4 (mins.)		30	45	60	90	120
Dry weight of sample (gms.)	i.e. W.	6.157	4.874	4.997	5.052	5.057
Volume of extract and washings(cc.)	V.	500	415	400	315	270
Aliquot taken for analysis (cc.)	A.	25	25	25	20	20
Wt. of ribose added (mg.)	R.	18.5	22.0	19.7	19.5	18.0

The weights of each sugar (mg.) extracted from the papers (average of duplicate chromatograms).

Sample		A	B	C	D	E
Galactose	x gal.	0.079	0.060	0.096	0.100	0.050
Glucose	x gluc.	0.041	0.034	0.051	0.066	0.043
Mannose	x man.	trace	trace	trace	0.008	0.018
Arabinose	x arab.	0.123	0.106	0.117	0.116	0.065
Xylose	x xyl.	0.102	0.093	0.128	0.180	0.146
Ribose	r.	0.290	0.247	0.264	0.251	0.099

The weights of each monosaccharide liberated per gm. original material during the time was calculated from:-

$$\text{weight of monosaccharide (mg.)} = x.\frac{R.V.}{r.A.} \cdot \frac{1}{W}$$

These weights were,

Galactose	16.4	18.2	22.9	24.2	24.3
Glucose	8.5	10.3	12.2	16.0	20.9
Mannose	trace	trace	trace	1.9	8.7
Arabinose	25.5	28.2	28.0	28.1	31.5
Xylose	21.1	25.2	30.6	43.6	70.9

The conditions for C were used in subsequent experiments.

2) Conditions for saccharifying the polysaccharides associated with lignin.

Three samples (approx. 1 gm.) of the lucerne pretreated for 1 hour on a boiling water bath with 1N H_2SO_4 (30 cc. per gm.) were hydrolysed with 72%(w/w) H_2SO_4 (15 cc. acid per gm. material) for 4 hours at 18±2°C., diluted to 1N H_2SO_4 (by addition of 23.5 cc. water for every 1 cc. acid used) and boiled under reflux for 1, 3 and 6 hours.

The acid-insoluble residue (mainly lignin) was filtered through an asbestos-lined gooch crucible and was dried overnight in the oven (at 80°C.) to constant weight. The crucible and lignin was carefully ignited with a Meker burner and reweighed. The difference in weight was the "ash-free lignin".

Aliquots from the filtrates (equivalent to about 100 mg. of the material hydrolysed with 72% acid) were neutralised ($BaCO_3$) after addition of a reference sugar (20 mg. approx. ribose). The sugars - glucose, xylose, mannose and ribose were separated on quantitative paper chromatograms (irrigated with solvent i).

A crude protein determination was carried out on a sample (50mg.) of the material and the ash content was determined on another portion (100 mg.) These were found to be 15.3% (N) and 0.5% (A) respectively.

The percentage recovery of carbohydrate (P) was calculated as follows.

Sample			1	2	3
Time refluxed with 1N H_2SO_4 (hours)			1	3	6
Sample weight	(gm.)	W	0.7635	1.0132	1.4092
Volume of filtrate	(cc.)	V	375	450	565
Aliquot taken	(cc.)	A	30	45	50
Wt. of ribose added	(gm.)	R	0.0194	0.0210	0.0176
Wt. of ash-free lignin	(gm.)	L	0.2310	0.2474	0.3056

The weights of sugars (mg.) found on the chromatograms (average of duplicate papers) were,

Glucose	m gluc.	0.489	0.421	1.367
Xylose	m xyl.	0.134	0.070	0.203
Mannose	m man.	0.041	0.057	0.031
Ribose	x	0.264	0.193	0.380

The results were expressed as polyarabinose, polyxylose etc. The weights of these "anhydro-sugars" were calculated using the factors $\frac{162}{180}$ for hexoses and $^{132}/_{180}$ for pentoses for conversion from the monosaccharides.

The total weights (gm.) of each anhydro-sugar (S) were calculated from

$$S = \frac{m.R.V.}{x.A} \cdot \frac{162}{182} \quad (\text{or } \frac{132}{150} \text{ for pentosans}).$$

Sample	1	2	3
S glucose	0.2425	0.4122	0.6436
S xylose	0.0650	0.0670	0.0935
S mannose	0.0203	0.0558	0.0146
Total	0.3278	0.5350	0.7517

The weight of the polysaccharide in the sample was found by correcting for the lignin, protein and ash contents, viz.

$$\frac{W\ (100-N-A)}{100} - L$$

and the percentage recovery (P) of the polysaccharide was calculated from

$$P = \frac{(S\ gluc. + S\ xyl. + S\ man.)\ 100}{\frac{W(100-N-A)}{100} - L.}$$

The recoveries in the samples refluxed for 1, 3 and 6 hours were 79.6%, 88.3% and 85.3% respectively. The conditions used for sample 2 were selected for the subsequent experiments.

3) Extraction of the water-soluble polysaccharides before 1N acid treatment.

Two samples (approx. 5 gm. each) of the lucerne were weighed

out.

Sample I was boiled under reflux with water (5 hrs.; 150 cc.). The residue was filtered and extracted again (4 hrs.; 150 cc.). The insoluble material was filtered off and thoroughly washed with water. The extract and washings were combined (volume 620 cc.) and an aliquot (310 cc.) was acidified (62 cc. of 6N H_2SO_4) and hydrolysed (5 hours boiling under reflux). Ribose (22.0 mg.) was added as a reference sugar, and the solution was neutralised ($BaCO_3$). The insoluble salts were filtered off and the solution was evaporated (30°C.; reduced pressure) and spotted onto two duplicate pairs of quantitative chromatograms run in solvents i and ii. From these the galactose, glucose, arabinose, xylose and ribose were estimated.

The residue was oven-dried and weighed. This was analysed by the methods described on pages 59 (sample C) and 62 (sample 2). The weights of sugars removed at each stage (calculated as anhydro-sugars) were expressed as percentages of the dry sample.

Sample II was analysed by the methods selected for the two-stage acid hydrolysis, viz. sample C (p. 59) and sample 2 (p. 62). [Full details of these two acid hydrolyses are described later in this thesis (pp. 73-76)]. As for sample I, the weights of anhydro-sugar were expressed as percentages of the original dry sample.

Sample		I	II
Water extract - Sample weight (gm.)		5.0	5.0
Sugars found on papers - galactose (mg.)		0.082	
glucose (mg.)		0.086	
arabinose (mg.)		0.186	
xylose (mg.)		0.030	
ribose (mg.)		0.186	
Weight of water extracted residue (gm.)		3.79	
Normal sulphuric acid extract.			
Sugars found on papers - galactose (mg.)		0.066	0.143
glucose (mg.)		0.027	0.078
arabinose (mg.)		0.127	0.286
xylose (mg.)		0.160	0.123
ribose (mg.)		0.290	0.596
Volume of extract (cc.)		440	500
Volume of aliquot analysed (cc.)		40	30
Weight of ribose added (gm.)		0.0181	0.0186
Weight after 1N extraction (gm.)		2.842	3.116
72% H_2SO_4 treatment			
Sample weight used (gm.)		0.8393	0.9461
Volume of filtrate (cc.)		420	410
Volume of aliquot analysed (cc.)		50	50
Weight of ribose added (gm.)		0.0280	0.0192
Sugars found on papers - glucose (mg.)		0.659	1.165
mannose (mg.)		0.028	0.039
xylose (mg.)		0.127	0.223
ribose (mg.)		0.371	0.412
Crude protein content of 1N residue (%)		13.58	13.68
Ash content of 1N residue (%)		0.23	0.34
Weight of ash-free lignin (gm.)		0.1967	0.2156

The following table shows the amounts of the polysaccharides removed during the separate stages of the analyses (calculated as percentages of the dry weight of the alcohol extracted plant material).

Sample	I				II		
Fraction	H_2O	1N	72%	Total	1N	72%	Total
Polygalactose	0.35	0.82	-	1.17	1.34	-	1.34
Polyarabinose	0.77	1.53	-	2.30	2.62	-	2.62
Polyxylose	0.12	1.93	5.47	7.52	1.13	6.04	7.17
Polymannose	-	-	1.23	1.23	-	1.08	1.08
Polyglucose	0.37	0.33	29.00	29.70	0.73	32.27	33.00

4) Comparison of the results obtained by the two stage acid hydrolysis with those from alkali extraction methods.

Three procedures were compared.

A) The two-stage acid hydrolysis (details pp. 73-76) of a sample of the lucerne (5.0 gm.).

B) Extraction of the polysaccharides from a sample (10 gm.) with:-

i) water. The sample was boiled under reflux for two periods (6 hours + 3 hours) with two portions of water (300 cc. each). The residue was washed with hot water. The extracts and washings were combined.

ii) ammonium oxalate (0.5% w/v). The residue from (i) was extracted for 3 hours + 3 hours on a boiling water bath with two 240 cc. portions. The two extracts and water washings were combined.

iii) sodium hydroxide (4% w/v). The residue from (ii) was shaken at room temperature for two periods (each 24 hours) with two portions (200 cc. each). The two extracts and final hot water washings were combined.

iv) The residue from (iii) was hydrolysed with 72% H_2SO_4 (p. 74).

C) Extraction of the polysaccharides from a sample (25 gm.) with:-

i) water. Conditions as for B(i), volume of water was 750 cc. each time.

ii) ammonium oxalate (0.5%). The residue from (i) was extracted for three periods (3 hours each) on a boiling water bath with three portions (250 cc. each) of oxalate. The three extracts and final water washings were combined.

iii) sodium hydroxide (16% w/v). The residue from (ii) was stirred continuously (in a N_2 atmosphere) for 48 hours with 520 cc. alkali. The insoluble residue was centrifuged down, separated and re-extracted (520 cc. alkali) for a further 48 hours under N_2. The residue was centrifuged down, separated, and washed with hot water in the centrifuge bottle and finally on a Buchner funnel. The extracts and washings were combined.

iv) The residue from (iii) was hydrolysed with 72% H_2SO_4 (p. 74).

Because of the large volumes of the extracts B(ii),(iii) and C(ii),(iii), it was necessary to concentrate them (30°C.; reduced pressure) before precipitating the polysaccharide with ethanol. (The sodium hydroxide extracts were acidified with glacial acetic

acid). Details of the concentrations and precipitation are tabulated.

	Concentrated to (cc.)	Volume of 95% Ethanol added (cc.)
B ii	250	500
B iii	225	450
C ii	300	600
C iii	850	1700

[On addition of further quantities of alcohol, only traces of precipitate were produced.] The precipitated polysaccharides were centrifuged down and washed in the centrifuge bottle with ethanol of increasing concentrations (80%, 90%, 95%) and finally with dry ether. These crude products were not reprecipitated and the fractions were dried (in vacuo) over P_2O_5. The yields were

B(ii)	0.989 gm.	C(ii)	3.1673 gm.
B(iii)	0.838 gm.	C(iii)	1.9041 gm.

Aliquots of B(i) and C(i) and portions (50-100 mg.) of B(ii),(iii) and C(ii),(iii) were hydrolysed by boiling under reflux with sulphuric acid (1N)for 5 hours. Ribose was added as a reference sugar and the sugars were estimated after paper chromatographic separation (solvents i and ii).

The analytical results (expressed as percentages of anhydro-sugars in the original alcohol extracted sample) are presented in the following table.

Sample	A			B					C				
	i	ii		i	ii	iii	iv		i	ii	iii	iv	
Extract	1N	72%	Total	H_2O	Ox.	4% NaOH	72%	Total	H_2O	Ox.	16% NaOH	72%	Total
Poly-galactose	0.77	-	0.77	0.39	0.14	0.22	-	0.75	0.21	0.15	0.48	-	0.84
Poly-arabinose	1.88	-	1.88	0.85	0.34	0.49	1.34	3.02	0.60	0.17	0.63	0.33	1.73
Poly-mannose	-	0.79	0.79	-	-	-	1.18	1.18	-	-	-	0.20	0.20
Poly-xylose	2.21	3.09	5.30	0.16	0.12	2.00	3.70	5.98	0.20	tr.	2.15	2.90	5.25
Poly-glucose	0.43	31.23	31.66	0.41	tr.	0.30	26.61	27.32	0.37	0.05	0.64	28.95	30.01
Total polysaccharide			40.40					38.25					38.03

Scheme developed for analysis of the carbohydrates in lucerne.

The fresh sample (30 gm. approx.) was placed in alcohol (50-60°C.) in a thermos jug as soon as possible after cutting.

Free sugar analysis.

The alcohol was decanted from the alcohol-immersed material. The sample was macerated in a fresh portion of alcohol (the stem, after cutting into 1" pieces), transferred quantitatively to a Soxhlet thimble (27x250 mm.)

and extracted with 80% ethanol (400 cc.) for 6 hours. All the alcohol solutions were combined and made up to a known volume. To an aliquot, equivalent to ca. 2 gm. dry lucerne, xylose (approx. 20 mg.) and an equal volume of water were added. The alcohol was evaporated (30°C.; reduced pressure) and the solution was concentrated to 100 cc. (approx.). An equal volume of water was added to this, and the solution was clarified by maintaining it at 95°C. for two minutes after the simultaneous addition of equi-molecular volumes of cadmium sulphate (10 cc.; 0.36N) and barium hydroxide solution. The mixture was thoroughly cooled and the insoluble salts were filtered off through a pad of supercel. The filtrate was electrodialysed, the voltage (ca. 130 v. d.c.) being adjusted, if necessary, to supply a current of not more than 1-1.2 amp (to avoid overheating).

After electrodialysis, the solution was evaporated (30°C.; reduced pressure) to about ½ cc. and approx. 0.03 cc. spotted onto each of duplicate chromatograms from a micropipette. The papers were irrigated with the organic layer of solvent i for 40 hours, dried in air and the side strips were cut off and sprayed with aniline oxalate.

A) The glucose, fructose, xylose and sucrose were eluted as described on pp. 37-38 and estimated by the Somogyi method.

B) The oligosaccharides were eluted by hanging the paper strip over 5.5 cc. boiling water for 1½ hours. The contents of the

tube were made up to 7 cc. and a 2 cc. aliquot was taken for a fructose determination by the colorimetric method (p. 43). Another aliquot was made 0.5N w.r.t. H_2SO_4 and heated under reflux on a boiling water bath for 4 hours. The solution was cooled, neutralised with 4N KOH (or NaOH) and the total reducing value determined by the Somogyi method.

To determine the ratio of sugar constituents in the oligosaccharides, most of the remaining ½ cc. of the sugar mixture was spotted onto a strip of Whatman 3MM chromatography paper (13 cm. x 50 cm.). This was irrigated for at least 40 hours with solvent i. The side strips were cut off, sprayed with aniline oxalate and the oligosaccharide portion was cut out and exhaustively extracted with water in a flask on a boiling water bath. The extracts were filtered and evaporated (30°C.; reduced pressure) to a syrup which was heated under reflux on a boiling water bath with H_2SO_4 (0.5N; 5 cc.) for 4 hours. The solution was cooled, neutralised (with $BaCO_3$) and filtered through supercel. The filtrate was evaporated (30°C.; reduced pressure) to a small volume (0.2-0.3 cc.). Any remaining precipitate was removed by a second filtration, and all the solution was spotted onto two duplicate chromatograms (Whatman No.1) which were irrigated with solvent ii for 40 hours. The glucose, fructose and galactose were estimated (and any arabinose or xylose if present). [Arabinose and fructose are not separated and the eluate is made up to a known volume e.g. 6 cc. An aliquot (2 cc.) was taken for a fructose determination by the

colorimetric method and another aliquot (3 cc.) taken for a total reducing value. By difference, the arabinose was estimated.]

Calculation. Applying a correction for 28% destruction of fructose under the hydrolysis conditions (see p. 43) to the fructose value, the ratio of sugars in the oligosaccharides was determined. From this ratio, the Somogyi factor for the total reducing value was calculated using the separate factors for the constituents. It was thus possible to convert the total reducing value in B to a weight of monosaccharide. The total weight of monosaccharide from the oligosaccharides was obtained after correcting for the fructose destruction during hydrolysis.

The percentage glucose, fructose, and sucrose in the dry lucerne was,

$$\text{weight on paper} \times \frac{\text{weight of added xylose}}{\text{weight of xylose on paper}} \times \frac{1}{\text{aliquot taken}} \times \frac{100}{\text{dry weight of sample}}$$

For the percentage oligosaccharide, the total weight of monosaccharide was multiplied by $\frac{162}{180}$ before substitution in the calculation.

Analysis of hemicelluloses, cellulose and lignin.

A) The alcohol extracted residue was dried in the oven (at 80°C.) overnight. It was hydrolysed with a measured volume of normal sulphuric acid for 1 hour on a boiling water bath (30 cc. acid per gm. dry weight). The insoluble residue was filtered, thoroughly washed with water and dried (after a final acetone

washing) at 80°C. overnight. [The acetone washings were discarded.] The extract and water washings were combined, made up to a known volume and an aliquot taken (equivalent to about 300 mg. of the weight of the residue before extraction). This aliquot was made 1N w.r.t. H_2SO_4 and boiled under reflux for 4 hours. The solution was cooled to room temperature and neutralised ($BaCO_3$) after the addition of ribose (approx. 20 mg.). The insoluble salts were filtered off on a pad of supercel and the solution, after concentration (30°C.; reduced pressure) to ½ cc., was filtered a second time if necessary. Exactly the same volume (ca. 0.03cc.) was spotted onto each of two duplicate pairs of chromatograms from a micropipette. One pair was irrigated (40 hours) with solvent i to obtain a clear separation of the reference sugar ribose. The other pair was irrigated with solvent ii to separate galactose, glucose, arabinose, xylose and ribose (the last two named overlap slightly). [The amount of the sugar thought to be rhamnose, which ran slightly faster than ribose on the papers, was very small and was not estimated.]

B) The residue after normal sulphuric acid treatment was dried and weighed. The ash content was determined on one portion (100 mg.) and the crude protein (total N x 6.25) on another (50 mg.).

A known quantity (1 - 1½ gm.) was hydrolysed with 72% w/w H_2SO_4 (15 cc. per gm.) for 4 hours at room temperature (18 ± 2°C.) with frequent stirring. The sulphuric acid was diluted to 1N by addition of 23.5 cc. water for every 1 cc. of 72% acid used, and

the solution was boiled for 3 hours under reflux. The insoluble lignin was filtered off through an unweighed asbestos-lined gooch crucible. The crucible was dried to constant weight (100°C.; overnight), carefully ignited with a Meker burner and its weight + ash determined. The difference in weight represents the "ash-free lignin".[30]

To an aliquot of the filtrate equivalent to about 100 mg. of the residue taken, ribose (ca. 20 mg.) was added. The solution was neutralised with barium carbonate, filtered and the filtrate evaporated to ½ cc. (30°C.; reduced pressure). The solution was filtered again (if necessary) to remove insoluble material, and the contents of 1 micropipette spotted onto each of duplicate chromatograms irrigated with solvent i. The glucose, mannose, xylose and ribose were estimated.

Calculation.

1st residue = residue after ethanol extraction.
2nd residue = residue after ethanol and 1N H_2SO_4 extractions.
Except where stated otherwise, weights are in grams.
G = dry weight lucerne sample.
w. = wt. 1st residue. W = do. 2nd residue.
f = fraction of hydrolysate of 1st residue taken. F = " " "
r = wt. ribose added to 1st residue hydrolysate. R = " " "
m = wt.(mg.) monosaccharides (on paper) from w. M = " " " (mg.)
x = wt.(mg.) standard ribose (on paper) from W. X = " " " (mg.)
A = % ash in 2nd residue.
N = % crude protein in 2nd residue.
H = wt. of 2nd residue hydrolysed.
L = wt. of ash-free lignin.

$$S = \text{wt. sugar from 72\% hydrolysis} = \frac{M.R}{X.F} \cdot \frac{162}{180} \text{ (or } \frac{132}{150} \text{ for pentosans).}$$

$$P = \text{\% recovery from 72\% hydrolysis} = \frac{(S\text{gluc.} + S\text{ xyl.} + S\text{ man.})100}{\frac{H(100-N-A)}{100}} - L$$

$$\% \text{ polysaccharide} = \left[\left(\frac{m.r}{x.f} \cdot \frac{162}{180}^{*} \right) + \frac{100\ S.W.}{P.H} \right] \frac{100}{G}$$

(* or $\frac{132}{150}$ for pentosans)

$$\% \text{ lignin} = \frac{100\ L.W}{H.G}$$

Seasonal variations of the carbohydrates in lucerne in 1954 and 1955.

The lucerne (du Puits variety) was sown on May 28th 1954 at the Bush Estate, Lothianburn, Midlothian, in a plot 9 yards x 8 yards in rows 1 foot apart. To reduce sampling errors, the plot was divided into 99 sections. As for the rye grass samples, the material from a number of these squares (5 for lucerne) was cut and thoroughly mixed before a random sample was taken for analysis.

The effect of sunshine on the amount of water-soluble carbohydrate in grass had been noted for perennial ryegrass (p.184) and in order to minimise any changes in carbohydrate content due to weather factors alone, no samples were taken on, or just after, a rainy or very dull day. Meteorological data were obtained from the sub-station at Bush House, a quarter of a mile from the plot.

Samples were taken every two or three weeks from 15th July until 16th November 1954. At the end of November the whole plot was cut back to just above ground level and the second growth sampled in 1955. No square which had been cut at any time during the first year for analysis was selected for sampling in the second

year. The sampling was started earlier in 1955, the first being taken on 24th May. Samples were afterwards gathered at intervals until 24th November and another was taken during the winter on 25th January 1956.

Two second growth samples were analysed in 1955 (samples 6 and 9). These represented the aftermath growth from samples 2 and 4 (respectively) which had been cut 11-12 weeks previously.

All the samples were taken about 11 or 12 o'clock in the morning to avoid diurnal changes, except Nos.1 and 8 in 1954 which were sampled at 2.30 p.m.

The samples were separated into leaf and stem in most cases and the samples for carbohydrate analysis were placed in hot alcohol within 30 minutes of cutting.

The carbohydrate constituents of the samples were determined using the method described on pp. 73-76 . Analysis of the flower and seed were carried out using the same conditions, although no preliminary experiments were carried out to ascertain if these analytical conditions were satisfactory.

The crude protein contents of oven-dried samples were estimated in 1954 and 1955 (p.45).

The ash, ether-soluble material and uronic anhydride contents were estimated on the oven-dried material of 1955 samples (p. 45).

All the analytical results for the seasonal variations cannot be included in this thesis and only the percentage compositions are tabulated. A specimen analysis is included to show how the results

PLANT- LUCERNE 3 STEM Dry weight 8·85 gm
Date- 5th JULY 1955.

N H_2SO_4 HYDROLYSATE

Wt. water-extd. res. = 6·34 gm
Vol. H_2SO_4 used = 190 cc
Total vol. of extract = 485 cc
Aliquot 25 cc

Add 3·04 cc 6N H_2SO_4 to aliquot.

Wt. w. glass = 5·9296 gm
+ ribose = 5·9503 "
Wt. ribose = 0·0207 "

Sugar.	c.c. thio.	net titre.	mg.
Blank (T&L)	10·06		
Galactose	8·57	1·49	0·229
Glucose	4·65	2·41	0·306
Arabinose	6·88	3·18	0·471
Xylose	7·40	2·66 & 2·90	0·394
Ribose	6·59	3·42 & 3·23	0·601
Blank (3-1-3)	10·06		
Ribose	6·83	3·23	

72% H_2SO_4 HYDROLYSATE. Wt. N H_2SO_4-extd. res. = 4·07 gm

Wt. crucible = 13·1862 gm.
+ residue = 13·8360 "
+ ash = 13·1864 "
Wt. residue = 0·6498 "
Wt. ash = 0·0002
% ash = 0·03

Wt. tube = 27·4558 gm
+ residue = 27·53466 "
Wt. res. = 0·0788 "
c.c. HCl = 3·43
Wt. C.P. = 4·29 mg
% C.P. = 5·44

Wt. tube = 15·281 gm
+ residue = 16·273 "
Wt. hydrolysed = 0·992 "
Vol. 72% H_2SO_4 = 14·68 cc
Diluted with 390 cc

Sugar.	c.c. thio.	Net titre.	mg.
Blank (3-1-3)	9·92		
Glucose	5·34	4·58	0·582
Mannose (contd)	9·40	0·52½	0·036
Xylose	8·96	0·96	0·131
Ribose	8·79	1·13	0·210

Wt. cruc. + lignin = 15·6048 gm
" ignited = 15·1652 "
Wt. ash-free lig. = 0·2396 "

Wt. w. glass = 5·9292 gm
+ ribose = 5·9518 "
Wt. ribose = 0·0226 "

72% H_2SO_4 filtrate
VOLUME 485 cc
ALIQUOT 50 cc.

$$S_{glucose} = \frac{0.582 \times 0.0226 \times 485 \times 162}{0.210 \times 50 \times 180} = 0.5128 \text{ gm}$$

$$S_{xylose} = \frac{0.131 \times 0.0226 \times 485 \times 132}{0.210 \times 50 \times 150} = 0.1129 \text{ gm}$$

$$S_{mannose} = \frac{0.036 \times 0.0226 \times 485 \times 162}{0.210 \times 50 \times 180} = 0.0317 \text{ gm}$$

$$0.6547 \text{ gm}$$

$$P = \frac{100 \times 0.6547}{0.992\left(\frac{100 - 5.44 - 0.03}{100}\right) - 0.2396} = 94.16\%.$$

$$\text{Lignin } \frac{100 \times 0.2396 \times 4.07}{0.992 \times 8.85} = 11.11\%.$$

$$\text{Polyarabinose } \frac{0.471 \times 0.0207 \times 485 \times 132 \times 100}{0.601 \times 25 \times 150 \times 8.85} = 3.13\%$$

$$\text{Polygalactose } \frac{0.229 \times 0.0207 \times 485 \times 162 \times 100}{0.601 \times 25 \times 180 \times 8.85} = 1.56\%$$

$$\text{Polyxylose } \left[\left(\frac{0.394 \times 0.0207 \times 485 \times 132}{0.601 \times 25 \times 150}\right) + \left(\frac{100 \times 0.1129 \times 4.07}{96.16 \times 0.992}\right)\right]\frac{100}{8.85}$$

$$(0.2317 + 0.4918)\,{}^{100}/_{8.85} = 8.17\%$$

$$\text{Polyglucose } \left[\left(\frac{0.306 \times 0.0207 \times 485 \times 162}{0.601 \times 25 \times 150}\right) + \left(\frac{100 \times 0.5128 \times 4.07}{94.16 \times 0.992}\right)\right]\frac{100}{8.85}$$

$$(0.1840 + 2.2350)\,{}^{100}/_{8.85} = 27.33\% \text{ (Glucosan } 2.08\%)$$

$$\text{Polymannose. } \frac{100 \times 0.0317 \times 4.07 \times 100}{96.16 \times 0.992 \times 8.85} = 1.52\%.$$

PLANT- LUCERNE SAMPLE 3 STEM.

Date - 5th JULY 1955
Conditions- PREVIOUS DAY - BRIGHT/SUNNY
SAMPLING DAY " "

Height = 20"
Wt. of 40 tillers = 35·4 gm
Dry wt./tiller = 892 mg

L/S 0·61 : 1

STAGE. JUST BEFORE FLOWERING.

MOISTURE

Wt. of dish = 176·5 gm
+ fresh grass = 217·2 "
+ dry grass = 188·5 "

Wt. of fresh = 40·7 gm
Wt. water lost = 28·7 "
% moisture = 70·5

CRUDE PROTEIN

Wt. bottle = 15·9091 gm
+ grass = 18·1032 gm
- N sample = 18·0431 gm

+ dry sample = 17·9629 "

Wt. wet N sample = 0·0601 gm
Wt. wet sample = 2·1340 "
Wt. dry sample = 2·0538 "
Dry wt. N sample = 0·0579 "

Titre = 3·76 cc
mg. N = 0·75
mg. C.P. = 4·69
% C.P. = 8·10

ALCOHOL EXTRACT.

Wt. of dish = 176·5 gm
+ sample = 206·5 "

Wt. fresh sample = 30·0 gm
Dry wt. sample = 8·85 "

Volume of extract = 1600 cc
Aliquot = 400 cc

Wt. w. glass = 5·9293 gm
+ xylose = 5·9502 "
Wt. of xylose = 0·0207 "

Sugar.		c.c. $Na_2S_2O_3$	Net titre	mg.	% dry weight.
Blank	(4 me reagent)	9·34			
Sucrose B	(6 me ")	14·43			
Oligos. B	(4 me ")	9·90			
Sucrose	6 me	3·84	10·56	1·343	3·21
Glucose	4 me	3·04	6·30	0·869	2·03
Fructose	4 me	3·14	6·14	0·851	1·99
Xylose	4 me	6·40	2·94	0·400	—
Oligos.	4 me	8·53	1·34	0·148	0·42.

WATER EXTRACT. Volume. Dilution. Vol. taken. Opt. density. mg. fructose. % fructosan

NOT CARRIED OUT ON LUCERNE SAMPLES.

were calculated (opposite).

In some cases the values for the whole plant, calculated from the leaf and stem results and the ratio of these two parts, are plotted on the graphs. In neither sample 4 or 5 in 1955 was the flower or seed plus case included in such a "whole plant" result i.e. in every case only the leaf plus stem was considered as the whole plant.

No correction was applied for the loss of fructose in the hydrolysis of the oligosaccharides of lucerne because of the very small quantities present. The ratio of the constituents was determined by separating and estimating the monosaccharides in the hydrolysate. The Somogyi factors for the oligosaccharides were not determined for every sample because it was found that the factor did not change greatly during the season. The ratio of the constituent sugars in a leaf sample was (as an example) galactose (1 part by weight), glucose (1.5 parts), fructose (1 part) and xylose (1 part) giving a Somogyi factor 0.136 while in the stem the ratio was galactose (1 part), glucose (7 parts), fructose (3 parts) and xylose (1 part) giving a factor 0.133. The slight variations found were 0.133 to 0.137 in the leaf, and 0.125 to 0.135 in the stem. A single value (0.135) was used to calculate the amount of oligosaccharides in all the leaf samples, and one value (0.130) for all the stem samples.

The wilting of lucerne.

A large sample of the lucerne was gathered at 11.30 a.m. on June 22nd 1955 from the plot at Bush Estate. The fresh material was used for sample 2 of the seasonal variations.

The remainder of the lucerne was brought back to the laboratory and spread out on the bench partially over tripod stands to allow easy access of air. Random samples were taken after 4, 8, 24, 53½ and 82 hours wilting and were analysed by the method described for the seasonal variations. Full polysaccharide analyses were not carried out, only the "glucosan" being estimated.

PART 1. DISCUSSION

Analytical Methods.

The development of paper and column chromatography during the last ten years has enabled chemists to separate satisfactorily, complex mixtures of chemical compounds. The technique has proved of immense value in carbohydrate research since it may be used to separate mixtures of sugars which may then be estimated accurately. Closely allied to these developments in chromatography have been the advances in the detection and estimation of microgram quantities of substances.

From this laboratory, a series of papers reporting "Analytical Studies on the Carbohydrates of Grasses and Clovers" has been published (58,59,60,82,83,151,152). The analytical methods have included the use of chromatography for the separation of the glucose, fructose, sucrose and oligosaccharides found in the 80% ethanol extract of the grasses and clovers, and the glucose, galactose, arabinose and xylose (also mannose and rhamnose on occasions) in the acid hydrolysates of the polysaccharides.

Plant enzymes can bring about changes in the carbohydrate constituents of herbage very rapidly (46,53), and it is essential to deactivate them as soon as possible after the plant is cut. The plant, after cutting, was separated into leaf and stem, and samples of these portions were immersed in hot alcohol. This method was considered (83) to give as nearly as possible the true composition of the living plant by destruction of the enzymes at the earliest possible time.

The free sugars (i.e. the mono-, di- and oligo-saccharides) were extracted in a Soxhlet apparatus with aqueous ethanol. In order to estimate the carbohydrates present, a reference sugar, not present in the extract, was added in known amount; (xylose was the sugar used). A large amount of material extracted simultaneously with the free sugars must be removed from solution before chromatograms can be run. The method for clarification was that selected by Laidlaw and Reid (82) who after testing several procedures, coprecipitated the contaminants with cadmium hydroxide and barium sulphate.

The presence of ions interferes with the chromatographic behaviour and their concentration must be kept to a minimum to avoid water-logging of the paper (30,48). Laidlaw and Reid found that deionisation could be effected either by ion-exchange resins or by electrodialysis, but Harwood (61) found that considerable washing was required to remove monosaccharides from Amberlite IR 4B resin, and that preferential adsorption of ribose occurred on Zeocarb 215 resin. In view of this tendency for adsorption, deionisation after clarification in the present work has been by electrodialysis in a perspex cell using parchment and formalised gelatin on a cloth base as cathode and anode membranes respectively. Although small amounts of sugars are lost by diffusion during the operation, the proportion of each sugar [except ribose (152)] is approximately the same, so that the method is applicable where xylose is added as a reference sugar. Ribose diffuses more rapidly. During the final

stages of the experimental work, in place of the membranes described above, sheets of ion-exchange resins were used because they had been shown (5) to possess a number of advantages viz.

(i) complete recoveries of sugars were obtained,
(ii) there was no danger of hydrolysis or epimerisation, and
(iii) less heat was generated because of the lower current passing than with the previous membranes. Solutions could thus be electrodialysed without danger of degradation of the sugar.

The separations have been carried out on Whatman No.1 chromatography paper, the most useful solvents being the organic phases of the mixtures: ethyl acetate-acetic acid-water and n-butanol-benzene-pyridine-water, although other solvents have been used for special purposes. The positions of the sugars were detected by spraying the paper or strips of the paper with aniline oxalate solution. Other spray reagents were used when specific carbohydrates or types of carbohydrates were to be identified. The use of a micropipette has made possible the preparation of replicate chromatograms for quantitative separations.

On some chromatograms, it was found that the solvent did not flow evenly down the paper, the spots near one edge running slightly faster than at the other, or the spots in the centre running slightly faster than those nearer the edges of the paper. This may cause slight overlapping of adjacent sugar zones but it is

unlikely that the errors would be large, the overlap only causing very slight variations in the proportions of the sugar constituents.

Some doubt had arisen about the destruction of sugars when they were washed off the chromatograms with hot water (81). However a comparative experiment showed that there was no evidence of any appreciable degradation. Since the elution with hot water was more rapid and convenient, its use was adopted in this work. The hot water elution technique was necessary to elute the oligosaccharides and the extraction of sucrose from the papers was combined with its hydrolysis by suspending the paper strip over boiling oxalic acid instead of water.

Several methods for the estimation of sugars after chromatographic separations have been tried by workers in this laboratory. In the recent work, however, the Somogyi method has replaced these, since it was found to give consistent and reliable results for the amount of sugar in the eluates from paper strips and also in carefully neutralised acid hydrolysates of sucrose, oligosaccharides and polysaccharides. The method (described on p. 40) was accurate to ± 5% and was used for aldoses and ketoses.

The estimation of oligosaccharides proved more troublesome particularly when fructose was a constituent sugar. The method entailed estimation of the total reducing power after hydrolysis. During hydrolysis, however, 28% of the fructose was destroyed (152) and a correction factor had to be introduced. In addition to this correction, the ratio of the constituent sugars had to be determined

in order to calculate the actual weight of monosaccharide which the total reducing value represented.

The method used for the estimation of the fructosan in ryegrass was the colour-producing reaction between hydrochloric acid, fructose and resorcinol, and could be used for the estimation of fructose or fructose units in polymers in very low concentrations.

The other plant polysaccharides were hydrolysed to monosaccharides which were separated on chromatograms and the amounts of each constituent were estimated. For these hydrolyses, sulphuric acid was used, since the ions in solution were readily removed (as barium sulphate) before chromatography.

Moisture determinations on herbage samples were carried out by oven-drying at 80°C., and this oven-dried material was used for the following determinations.

(i) The total nitrogen content (estimated by the micro-Kjeldahl method) was used to calculate the crude protein content.

(ii) The ash content (determined by ignition of a sample).

(iii) The ether-soluble material (by shaking with anhydrous ether).

(iv) The total uronic anhydride contents of the lucerne samples (in 1955) were determined from the amount of carbon dioxide liberated on decarboxylation. No corrections were applied for small amounts (ca. 0.5%) of carbon dioxide liberated from other carbohydrate residues e.g. cellulose, starch, sucrose or monosaccharides (21) nor was any allowance made for the decarboxylation of any other organic acid (85). Grasses and lucerne do not contain

free uronic acids, and the results have been reported as percentages of uronic anhydride. This includes all the uronic acid-containing constituents viz. pectic substances and any uronic acids present in the hemicelluloses. Since the uronic acid content of the latter only accounts for a small proportion of the uronide content (the uronic anhydride contents were 11.2% and 3.1% before and after extraction with ammonium oxalate - p. 153), the changes found in the results of this estimation can be attributed to those occurring in the amount of pectic materials.

Analysis of Perennial Ryegrass - 1953.

The analytical methods for the determination of the carbohydrate constituents had been developed before the author began the work reported in this thesis. He analysed the aftermath (i.e. second growth) samples of the grass; Dr. Clare B. Wylam had analysed the first growth. The results are reported and discussed in a paper (reproduced on pp. 179-196) which has been accepted for publication (88).

The results of the author's contribution to this study (see table, p. 87) agreed well with the trends expected from the study of the first growth. The lack of active growth (the height was only 10 inches) was reflected in the low cell-wall polysaccharide contents. The hexose, sucrose and oligosaccharide contents were

similar to those found in the first growth. The fructosan content of the stem had risen to 17% in the last sample taken and it is probable that this was due to the small demand for carbohydrate for the synthesis of cell-wall material. Such a trend for higher fructosan values had been shown by Waite and Boyd during a study of the carbohydrates of S23 perennial ryegrass which was cut every time it reached the height of 8-10 inches (136). In the Autumn, when the growing point remained vegetative (see p.56) and more time elapsed between cuttings due to slower growth, the fructosan contents were slightly higher.

The conclusions reached from the study of the first and the aftermath growths were:

i) No major seasonal trends were evident in the amounts of glucose, fructose and sucrose.

ii) The plant accumulated in the stem a reserve of fructosan which reached a maximum (21% of the dry weight) before diminishing in the autumn.

iii) There was a gradual increase in the percentages of cell-wall polysaccharides.

The most interesting feature was the relation between the fructosan accumulation and the synthesis of cell-wall material during the plant's growth. At times when the grass showed obvious demands for carbohydrate viz. at periods of rapid stem growth or of flower and seed development, it appeared that this was at the expense of the reserve fructosan which accumulated if the plant was relatively inactive but decreased (in percentage) at those other periods of the plant's development.

Date	15th Sept.	29th Sept.		14th Oct.		28th Oct.		11th Nov.	
Part of plant	L and S	L	S	L	S	L	S	L	S
L/S ratio	3.2/1	3.1 : 1		2.9 : 1		3.5 : 1		2.8 : 1	
Crude protein	14.8	20.7	10.9	13.7	9.7	16.4	9.4	16.5	9.3
Glucose	0.4	0.2	2.3	0.7	1.2	0.3	1.1	0.3	1.1
Fructose	0.5	0.5	2.8	1.1	1.2	0.6	1.2	0.6	1.4
Sucrose	2.5	1.6	4.6	3.4	4.0	6.1	4.0	5.1	5.0
Oligosaccharides	4.9	1.2	7.1	2.7	5.1	2.8	4.6	1.6	3.3
Fructosan	2.0	3.0	13.0	2.0	14.0	4.0	13.0	6.0	17.0
Polygalactose				0.7	0.9			0.9	1.1
Polyarabinose				1.9	2.8			2.3	3.0
Polyxylose				5.3	8.3			6.4	8.5
Cellulose				16.2	18.2			18.9	18.9
Lignin				3.0	2.4			4.8	4.2

Analysis of Perennial Ryegrass (including Roots) - 1955.

After the study of the seasonal variations of the carbohydrates in the aerial portions of perennial ryegrass, it was thought desirable to extend the study to include the changes simultaneously occurring in the roots. As for the above ground material, the work carried out by previous workers has involved the use of the general methods of agricultural chemistry described in the introduction, and it was hoped that by applying the new techniques

developed for the leaf and stem of grass, to obtain more significant information about the changes in the amounts of the individual carbohydrates contained in the roots.

The major difficulty in the study of the roots of any plant is the problem of obtaining a satisfactory sample for quantitative analysis. Much soil is attached to the roots, and the removal of all the very fine particles adhering to the root-hairs is a very difficult, if not impossible, task. The result is that even with very thorough washing in running water, the ash was found to account for as much as 15% of the dry matter. Such exhaustive washing will also remove small amounts of the water-soluble sugars.

In order to avoid the very fine particles found in soil, the grass for this experiment was grown in a mixture of a coarse and a fine sand. The coarser the sand, the more easy and efficient is its separation from the roots but such a medium will not hold sufficient moisture for the plant's requirements. For this reason, a quantity of fine sand was thoroughly mixed with it. Even with this mixture, it was necessary to water the grass daily.

For sand culture, an adequate supply of inorganic nutrients for the growth of the plant must be provided. The daily watering washed away the nutrients supplied and regular applications of fresh solutions were necessary.

Hydroponics, the growth of plants in a purely liquid medium, was not used although it would have been the most convenient method.

The reason for this was that it was desired that the grass should be grown under conditions reasonably similar to those found naturally.

When sampling, the bulk of the sand was shaken off the roots by hand, but this still left a large amount of adhering material, and several liquids were tried for the removal of this remaining sand. Water permitted easy removal of most of this contaminant but also removed some of the sugar and the sample left was very wet. The problem of satisfactory subsampling for moisture and analysis determinations was considerable. Acetone was found to be suitable since it allowed very easy removal of most of the sand, and using a cold air blast, the excess liquid could be removed in two or three minutes. As with the water washings, however, small quantities of sugars were extracted.

After washing with acetone, two subsamples of the root material were weighed out. One was used for the ash determination and the other (for carbohydrate analysis) was placed in hot alcohol to deactivate the enzymes. The free sugars (i.e. mono-, di- and oligosaccharides) could be extracted from the macerated material with 80% ethanol in a Soxhlet apparatus. Because the acetone washings were found to contain small quantities of the alcohol-soluble sugars, an aliquot, corresponding to the proportion of the washed sample taken for analysis, was added to the alcohol extract from the root, and the solution was analysed by the method described on p. 46 .

The polysaccharides in the roots were analysed using the same conditions as for the rest of the plant (p. 47) except that heating on a boiling water bath for 1¼ hours (not 1 hour) was required for complete removal of all the arabinose-containing material.

Samples of the grass were taken at intervals during the season and the carbohydrates in the roots and the aerial portions estimated. No separation into leaf and stem was carried out. Throughout the analyses, the ash contents of the roots were high and this factor was a possible source of error where subsamples were taken for analysis. The dry ash-free weight was the only convenient basis on which to calculate a percentage composition. This may have introduced another unavoidable error in view of the probable change in the ash content of the roots with maturity (such as occurs in the leaf and stem). A further sampling error may arise by virtue of the small size of the samples taken for analysis. The grass from one half of an orange box was separated and a random portion of this taken for analysis. By this method no allowance could be made for uneven growth in the different sections of the boxes and in order to minimise this, those sections where growth appeared poor, were not used during sampling.

Examination of the growing points of the grass showed that they did not change from the vegetative to the floral state at any time during the season (see p. 56).

Date
31·5·55
15·6·55
5·7·55
27·7·55
6·9·55
27·9·55
11·10·55
No.4.
Hours
40
32
24
16
8
3
1
Day
No.3.
No.2.
No.1.
% Dry ash-free weight
1
2
3
4
5
6
7
Aerial portions of plant
Roots
Whole plant (calculated)

Changes in the carbohydrate composition.

It is not possible to include all the analytical results in this thesis and only the percentage compositions are shown in the table (overleaf). The results for the whole plant may be calculated from the ratio of roots to aerial portions.

Water-soluble carbohydrates. The amount of glucose plus fructose (graph 1) in the root was small (less than 1%) and showed only slight fluctuations, whereas in the above ground portion there was a very slight decrease over the season.

The percentage of sucrose plus oligosaccharides above ground (graph 2) did show a general downward trend but there was a peak about mid-season (July 27th) corresponding with a period of bright sunshine (graph 4). In the roots, however, the amount of these components did not follow the sunshine closely. Between samples 2 and 3 and between 5 and 6, there were marked increases in the percentage but a small decrease in both sucrose and oligosaccharides was detected during the intermediate period.

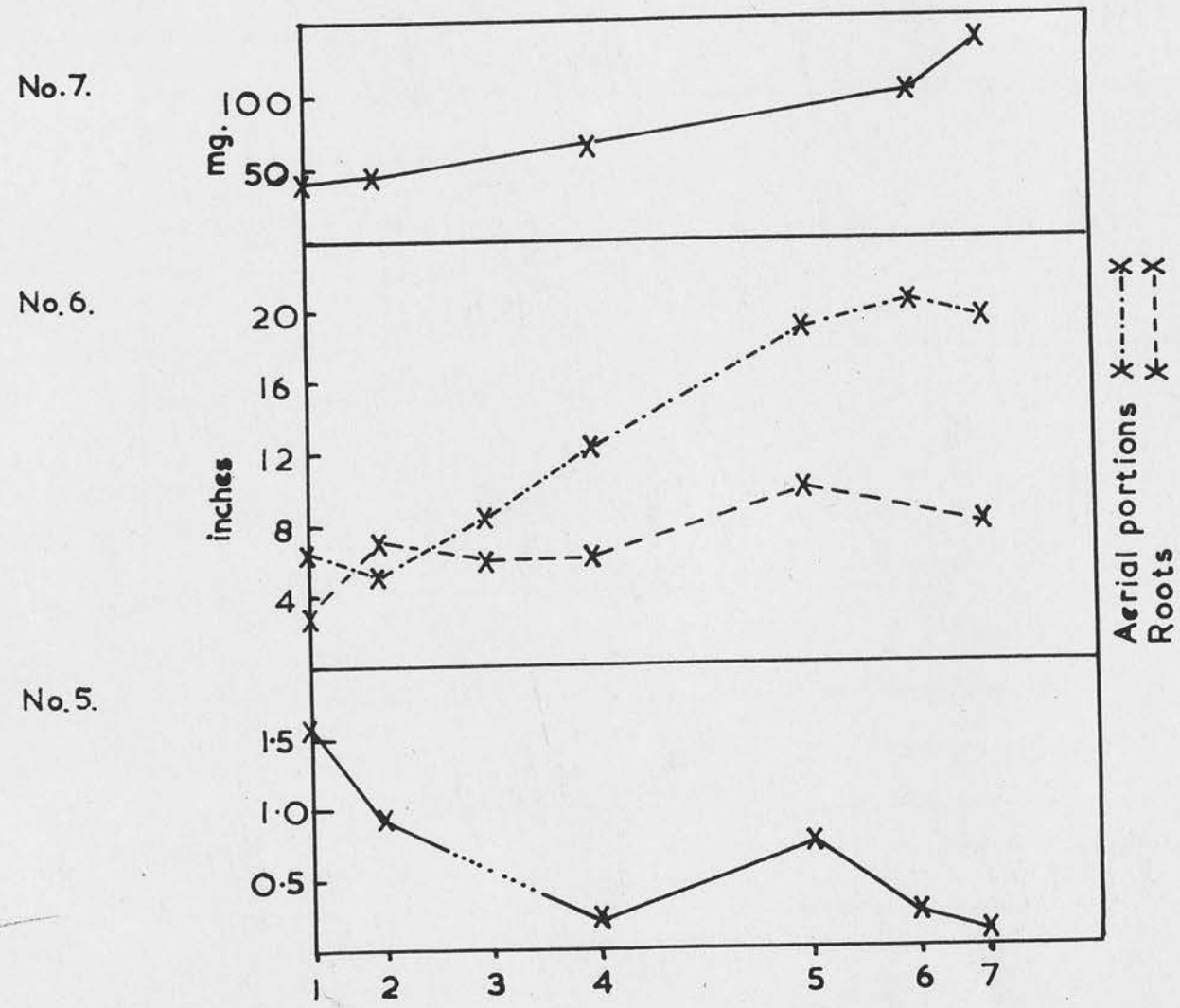
The fructosan content of the aerial parts (graph 3) showed a broad maximum, followed by a very marked decrease between samples 4 and 5. In the roots, the fructosan content showed a peak at July 27th (sample 4) followed by a decrease at the same time as the decrease in the above ground portions.

The ratio root:aerial portions, the lengths of these two portions and the dry weight per tiller are given in graphs 5,6 and 7 respectively.

Table 2.

Sample	1	1	2	2	3	4	4	5	5	6	6	7	7
Part of Plant	Top	Root	Top	Root	Root	Top	Root	Top	Root	Top	Root	Top	Root
Glucose	1.03	0.53	0.56	0.17	0.49	0.88	0.46	0.57	0.39	0.63	1.05	0.28	0.46
Fructose	1.27	0.42	1.02	0.20	0.42	0.88	0.30	0.73	0.31	0.65	0.62	0.24	0.39
Sucrose	3.31	1.79	1.98	0.20	2.12	3.39	1.86	0.92	1.52	1.16	3.41	1.37	0.93
Oligosaccharides	0.60		0.09	0.03	0.58	1.05	0.56	0.20	0.17	0.17	0.60	0.43	0.19
Fructosan	1.83	0.27	5.78	0.20	0.28	6.49	1.72	1.58	0.64	1.57	1.15	2.25	0.97
Total water-soluble carbo-hydrate	8.04	3.01	9.43	0.80	3.89	12.69	4.90	4.00	3.06	4.18	6.83	4.57	2.94
Polyarabinose	2.24	1.07	2.14	1.15	2.36	3.63	3.50	Analysis sample lost	2.94	2.27	4.93	4.24	2.78
Polygalactose	0.83	0.65	0.96	0.88	1.46	1.05	1.34		0.54	1.29	3.52	1.82	1.48
Polyxylose	5.34	4.02	4.70	3.56	9.67	6.78	19.44		12.17	9.51	15.17	9.42	9.34
Cellulose	16.09	10.55	14.33	6.99	15.55	22.43	44.35		22.33	22.31	22.70	23.96	15.75
"Lignin"	3.02	4.60	3.70	3.77	5.82	4.13	25.18		13.14	6.01	14.17	5.18	8.03
Ratio of parts	1 : 1.55		1 : 0.92		-	1 : 0.22		1 : 0.75		1 : 0.25		1 : 0.10	

Crude protein 5 root 10.5% 7 root 15.8% (% dry ash-free weight)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7
Aerial portions
Roots

As found in the seasonal study in 1953, it has been possible to relate major changes in the water-soluble carbohydrates to climatic factors and the rate and stage of growth. From the changes in the percentages of sugars, it is convenient to divide the season into four periods (i) up to 15th June, (ii) 15th June to 27th July, (iii) 27th July to 6th September and (iv) after 6th September.

From the growth curves, it can be seen that there was a fall in the ratio of below- to above-ground portions in the early part of the season (graph 5), indicating that a greater weight of top than root was being synthesised. No significant increase in the average weight per tiller was evident (graph 7) nor was there any increase in height (graph 6). The only explanation of these facts is that there is an increase in the number of tillers put forth by the plant. Troughton (131) and Cooper (31) have reported an increase in the number of tillers of perennial ryegrass during the first 60-100 days of its growth. June 15th was approximately 70 days after sowing and thus it is probable that until this time, the plants were producing new tillers in appreciable quantities. This increased amount of young leaf provides a large surface area for photosynthesis which will explain the increases in the amount of fructosan in the shoots.

During the second stage, there was a continued fall in the ratio root:above-ground material. The aerial portions were increasing in height while the length of the roots was practically unchanged. This increase in height gave rise to a slight increase in tiller weight. It is thought that the plant's energy requirements for tillering were reduced at this stage, and although the grass was increasing in height, there was abundant sunshine for photo-synthesis (graph 4). During this period there was little increase in the percentage of fructosan above ground and it appeared that the carbohydrate being synthesised was not stored in the aerial portions but was translocated to the root system where increases in the amounts of fructosan, sucrose and oligosaccharides were evident.

Later in the season (July 27th to September 6th), there was an increase in the proportion of roots being formed. This was taking place at a time of poorer weather with less sunshine and the plant may have been unable to produce enough carbohydrate. There would thus be a tendency for the water-soluble carbohydrate in the roots to be used for conversion to structural material in the developing root resulting in decreased percentages.

By the end of September, vertical plant growth had almost ceased in both over- and under-ground parts. The rapid increase in tiller weight found was confirmed by the decrease in the ratio of the root:aerial portions indicating that the leaf and stem, although not increasing in length, were increasing in dry weight. The slight fluctuations found in the amounts of the water-soluble carbohydrates in the root, follow the curve of the sunshine,

i.e. the excess carbohydrate not required immediately for synthesis in the leaves and stems appeared to be, temporarily at least, accumulated in the roots.

Polysaccharide constituents. The changes in the amounts of polysaccharides in both the portions of the grass were similar. A very slight increase (2% to 4%) was evident in the amount of polyarabinose and polygalactose.

In the roots, the polyxylose and cellulose changes indicated a gradual increase over the season. The same was also evident for the polyxylose in the aerial portion although the cellulose content of that part of the plant reached a maximum fairly early in the season, i.e. the xylose content of the cellulosic material increased with age (as found in the seasonal study in 1953).

In the study of the roots, although full analyses have not been carried out, it appears that much of the ash-free material has not been estimated. Crude protein determinations were not made regularly; in two samples the order of the crude protein content was 10-15%. There are few sources of large errors in the estimation of the ethanol soluble carbohydrates, but the accuracy of the polysaccharide results is not so certain due to the probability of uneven subsampling of materials of such high ash contents.

Further work will be necessary on the preparation of samples with as low an ash content as possible before more reliable results can be obtained. It would also be an advantage to find a method of disintegrating the roots more thoroughly since their tough nature tends to make them rather resistant to hydrolysis.

Development of a method for the analysis of the carbohydrates in lucerne.

Following the study of the monocotyledon, ryegrass, it was decided to examine the carbohydrates in the dicotyledon, lucerne (Medicago sativa). For a preliminary examination, the conditions for the analysis of the ryegrass were used.

The results of this examination showed that the same mixture of sugars as from ryegrass were extracted with 80% ethanol. Shaking the residue with cold water did not remove any single polysaccharide, but a small amount of a mixture which on hydrolysis yielded galactose, glucose, arabinose and xylose. The normal sulphuric acid treatment on a boiling water bath dissolved most of the hemicellulose components containing the same four sugars found in the aqueous extract. The residue was dissolved in 72% sulphuric acid and after hydrolysis, was shown to contain mainly glucose and xylose although there was some mannose and galactose. From these observations, it was thought that the scheme for the analysis was satisfactory provided the conditions were adequate for extraction and hydrolysis of the various carbohydrate constituents. The following experiments were carried out to test their suitability.

It was found that the time of 6 hours was adequate for the removal of the free sugars in the Soxhlet apparatus.

In the two-stage acid hydrolysis method for estimating plant polysaccharides, it is desirable to separate as much as possible of

the hemicellulose from the cellulose to avoid decomposition losses during the 72% sulphuric acid hydrolysis. Normal sulphuric acid was used for this pretreatment. The results showed that the time 1 hour was most convenient. Within this time, practically all the polygalactose and polyarabinose material has been dissolved. The xylan was not completely removed in this time; the amount extracted bore an approximately linear relation to the time the residue was in contact with the acid.

Examination of the literature (6,112) showed that mannans were resistant to hydrolysis with dilute acids, and therefore it was not desirable to prolong the extraction time beyond 1 hour to avoid dissolution of the mannose-containing material. If this was brought into solution by the dilute acid, subsequent hydrolysis for 4 hours might not give a quantitative hydrolysis. The polymannose was hydrolysed with the 72% acid.

The possibility that some destruction of the monosaccharides might occur during the time of hydrolysis was studied by Harwood (58) who showed that such decomposition did not exceed 6%. Boiling for 4 hours under reflux was found to be adequate for complete hydrolysis; as in the analyses of ryegrass hemicellulose, no significant amounts of partially hydrolysed oligosaccharides were detected on the chromatograms.

For the hydrolysis of the cellulose-containing residue of ryegrass, 72% sulphuric acid had been selected because there was no

evidence of large sugar losses due to degradation (58), and this acid was chosen for the hydrolysis of the lucerne residue. Treatment for 4 hours at room temperature, followed by dilution (to 1N H_2SO_4) and boiling for 3 hours were found to be the best conditions. The acid-insoluble residue (mainly lignin) was determined by ignition and the monosaccharides in the hydrolysate were estimated after separation on a chromatogram.

It was realised that this analytical method would not give a true lignin value (58) due to contamination (by protein), but the amount of acid-insoluble residue can be used to show the trends of lignification in lucerne.

Having provisionally selected the conditions for the acid hydrolyses, two further experiments were carried out. The first showed that there were no advantages in extracting the lucerne with water before treating it with dilute acid; the amount of carbohydrate removed was small (ca. 4% of the total polysaccharide) and only represented a portion of the material which would be estimated in the first acid hydrolysate.

The second experiment showed that the results obtained by the two-stage acid hydrolysis were comparable with those found after separation and hydrolysis of the various polysaccharide fractions. A sample of lucerne was extracted successively with boiling water, ammonium oxalate and sodium hydroxide. The polysaccharides extracted in these fractions and those in the insoluble residue were estimated after hydrolysis. The results showed good agreement

for the amount of polysaccharide in the different samples (p. 70). Slightly lower results were obtained in the four-stage analyses probably by mechanical loss where the isolation of products was involved, and loss due to small amounts of material soluble in the 65% ethanol used to precipitate the polysaccharide. Variations in the amount of each anhydro-sugar between the samples may to some extent be due to slight differences in the physical states of the polysaccharides due to the treatments prior to their hydrolyses (since the extraction conditions were different in each case).

The polyglucose value for the material insoluble in the 1N sulphuric acid under the experimental conditions is approximately the same as that found in the fraction which will consist mainly of cellulose (p. 70 ,fraction C(iv)) since by definition α-cellulose is the material insoluble in 17% sodium hydroxide (if the extraction is carried out in a nitrogen atmosphere). In the analyses, therefore, the glucose found in the 72% sulphuric acid hydrolysate represents (approximately) the cellulose content of the lucerne.

It was concluded from these experiments that the two-stage acid hydrolysis of the alcohol-extracted lucerne afforded a convenient method for the analysis of the total amount of each constituent of the polysaccharides and it was therefore applied to samples of lucerne in order to obtain a picture of the changes in composition occurring during growth.

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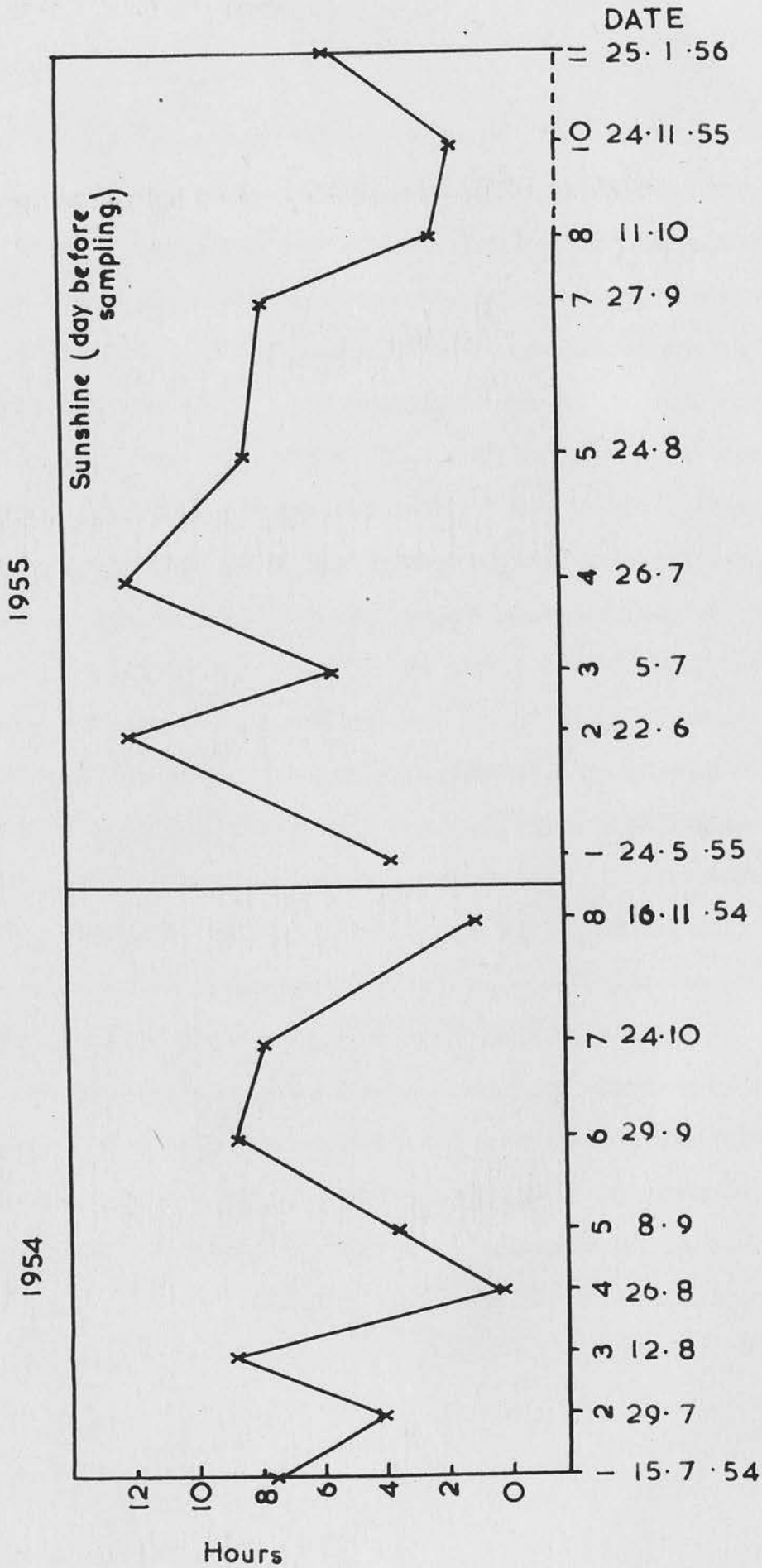

DATE
11 25·1·56
10 24·11·55
8 11·10
7 27·9
5 24·8
4 26·7
3 5·7
2 22·6
– 24·5·55
8 16·11·54
7 24·10
6 29·9
5 8·9
4 26·8
3 12·8
2 29·7
– 15·7·54
1955
1954
Sunshine (day before sampling.)
12
10
8
6
4
2
0
Hours

Analysis of lucerne during 1954 and 1955.

Table 3 gives the details of sampling, the height of the lucerne and the leaf:stem ratio of the dry plant. The analytical results for the carbohydrates, crude protein, uronic anhydride (all expressed as percentages of the dry weight) are contained in tables 4 and 5, and the ash and ether-soluble material contents in table 6.

The expression of results as percentages of the dry weight is the most convenient method although some changes may be masked because percentages of other constituents also vary. The percentage composition gives the concentrations of each component in the plant, but does not represent the actual quantities involved. In some cases, a constant and even a decreasing percentage may hide an increase in absolute quantity because of gains in other constituents.

Meteorological data are shown in table 7. The weather during the two years was very different. The 1954 summer (i.e. the first year) was poor, little sunshine and a moderately high rainfall, and this contrasted with the unusually dry summer of 1955 when there were considerable amounts of sunshine.

As can be seen from graph 8, most of the samples had at least three hours of sunshine on the day previous to sampling. It was unlikely therefore that any abnormal conditions due to lack of sufficient sunshine for photosynthesis (such as found in the shading experiment with ryegrass, p.184) would be found during either season.

Table 3.

Sample	Date	Parts of plant sampled	Height (inches)	Ratio L:S:other parts	Notes
1954					
1	15 July	Whole	3	Not detd.	
2	29 July	Whole	6-8	Not detd.	
3	12 August	Whole	9-10	Not detd.	
4	26 August	Whole	12-14	Not detd.	
5	8 September	Leaf+Stem	14-16	0.80	Just preflowering stage in a few plants
6	29 September	Leaf+Stem	18	0.77	
7	20 October	Leaf+Stem	18	0.58	
8	16 November	Leaf+Stem	18	0.45	
1955					
1	24 May	Leaf+Stem	9	0.90	
2	22 June	Leaf+Stem	18	0.73	
3	5 July	Leaf+Stem	20	0.61	Just preflowering stage
4	26 July	Leaf+Stem +Flower	30	L S F 0.38: 1 :0.15	Fully flowered
5	24 August	Leaf+Stem +Seed	30	L S Seed 0.45: 1: 0.43	Seeds formed
6	7 September	Leaf+Stem	18-24	0.41	Aftermath - 11 weeks after sample 2
7	27 September	Leaf+Stem	30	0.28	
8	11 October	Leaf+Stem	30	0.17	
9	19 October	Leaf+Stem	20	0.67	Aftermath - 11 weeks after sample 4
10	24 November	Stem	30	Not detd.	Very few leaves left on plant
11	1956 25 January	Stem	30	Not detd.	No leaves on plant. Weather very cold.

Table 4.

	1	2	3	4	5L	5S	6L	6S	7L	7S	8L	8S
Moisture content	73.0	81.0	82.2	83.9	84.9	80.0	77.7	72.7	76.9	81.3	71.5	69.7
Crude protein	22.3	21.2	19.9	21.5	30.4	9.1	26.5	9.4	25.5	10.4	22.6	9.3
Glucose	0.33	0.47	0.53	0.62	1.07	1.19	1.35	1.20	0.30	1.14	0.65	0.45
Fructose	0.29	0.48	0.57	0.78	0.42	1.29	0.64	1.70	0.40	1.87	1.18	0.64
Sucrose	1.01	1.23	1.97	1.94	1.26	3.14	1.98	5.55	2.38	5.91	3.19	6.89
Oligosaccharides	0.34	0.84	0.77	0.59	0.76	0.43	0.93	0.42	0.41	0.58	1.18	0.99
Carbohydrate in 80% alcohol extract	1.97	3.02	3.84	3.93	3.51	6.05	4.90	8.87	3.49	9.50	6.20	8.97
Polyarabinose	2.31	2.92	1.26	2.37	2.18	2.09	2.19	2.33	1.50	2.59	2.45	2.75
Polyxylose	1.91	2.74	2.85	2.62	3.22	10.73	1.24	6.18	3.50	8.77	3.23	8.48
Polygalactose	1.26	1.20	0.66	1.24	0.92	0.30	1.03	1.27	1.07	2.20	1.29	1.07
Polymannose	0.69	0.97	1.46	0.78	0.61	1.55	0.28	0.60	0.69	2.37	0.58	1.94
Polyglucose	17.11	19.60	16.48	17.68	8.36	31.42	7.83	24.40	6.70	27.70	7.64	33.32
"Glucosan"	9.50	9.19	2.15	1.54	1.85	1.35	1.94	0.73	1.50	1.50	0.78	0.57
"Lignin"	5.2	3.7	8.4	9.3	6.0	13.7	7.5	12.6	6.9	18.2	7.6	18.7

L = Leaf Sample. S = Stem Sample.

Results of seasonal study - 1954 (Lucerne).

All results as percentages dry weight (except moisture).

Table 5 (part 1).

	1L	1S	2L	2S	3L	3S	4L	4S	4F	5L	5S	5Seed
Moisture content	76.3	81.9	75.6	75.7	72.9	70.5	71.3	65.8	76.1	70.5	61.5	72.0
Crude protein	32.2	22.3	31.1	10.7	29.2	8.1	22.0	6.9	24.5	19.6	6.3	25.9
Glucose	0.57	1.81	0.57	2.60	0.30	2.03	1.65	1.85	3.07	0.22	0.48	1.06
Fructose	0.64	1.66	0.62	2.03	0.25	1.99	0.49	1.60	3.52	0.15	0.68	1.39
Sucrose	1.42	1.67	1.21	2.13	0.81	3.21	0.41	3.35	1.67	0.62	1.32	1.93
Oligosaccharides	0.10	0.01	0.13	0.40	0.24	0.42	0.35	0.20	0.21	0.49	0.15	0.63
Carbohydrate in 80% ethanol extract	2.73	5.15	2.53	7.16	1.60	7.65	2.90	7.00	8.47	1.48	2.63	5.01
Polyarabinose	4.39	3.17	3.45	2.45	1.77	3.13	2.00	2.07	4.88	1.71	2.88	3.40
Polyxylose	3.63	6.37	1.54	8.85	3.53	8.17	4.13	8.88	4.36	9.48	11.10	9.04
Polygalactose	3.72	1.30	0.89	1.34	1.03	1.56	0.42	0.77	2.05	1.53	1.53	1.35
Polymannose	0.56	0.73	1.13	1.79	0.38	1.56	0.31	2.17	1.58	0.50	1.68	1.45
Polyglucose	16.55	21.68	17.00	26.80	15.22	27.32	17.49	38.71	10.87	16.02	33.70	14.40
"Glucosan"	4.75	0.88	11.16	0.78	9.29	2.08	2.58	5.73	2.75	4.02	0.79	1.18
"Lignin"	3.50	6.43	7.60	10.60	4.82	11.11	6.12	16.86	5.54	7.32	14.26	8.85
Uronic Anhydride	12.25	9.29	10.83	8.78	N.D.	N.D.	11.66	6.56	11.54	10.52	7.68	28.43

L = Leaf Sample. S = Stem Sample. F = Flower Sample.

Results of seasonal study - 1955 (Lucerne).

N.D. = Not determined.

All results as percentages of the dry weight (except moisture).

Table 5 (part 2).

	6L	6S	7L	7S	8L	8S	9L	9S	10S	11S
Moisture content	70.7	63.5	69.1	62.0	71.2	62.0	69.3	67.0	51.2	21.4
Crude protein	20.3	6.4	18.6	5.5	16.8	5.2	24.7	8.0	3.6	3.0
Glucose	0.12	0.45	0.19	0.18	0.11	0.19	0.48	0.45	0.09	
Fructose	0.10	0.62	0.14	0.22	0.10	0.28	0.67	0.56	0.07	
Sucrose	1.79	2.74	1.63	2.04	1.20	1.91	2.22	5.56	0.12	
Oligosaccharides	0.26	2.48	0.19	0.22	0.42	0.15	0.11	0.30	0.11	
Carbohydrate in 80% ethanol extract	2.27	6.29	2.15	2.66	1.83	2.53	3.48	6.87	0.39	
Polyarabinose	1.68	3.72	2.22	1.77	1.86	2.30	2.18	1.92	2.71	1.40
Polyxylose	1.24	9.95	2.48	6.60	3.23	11.73	1.40	9.52	15.28	8.49
Polygalactose	1.00	1.46	0.85	0.60	0.66	1.04	0.72	1.59	1.76	0.90
Polymannose	0.72	1.70	0.30	2.35	0.85	3.02	0.36	1.15	3.72	2.50
Polyglucose	10.12	34.49	9.81	33.96	10.82	31.94	7.62	28.73	27.75	43.64
"Glucosan"	2.47	1.39	1.72	0.25	1.28	0.39	1.65	0.58	1.49	0.52
"Lignin"	6.68	14.55	6.01	15.61	5.22	15.62	7.59	12.24	15.40	19.61
Uronic Anhydride	11.87	9.80	N.D.	N.D.	9.88	7.44	N.D.	N.D.	5.21	N.D.

Only traces of sugars present. (Not estimated).

L = Leaf Sample. S = Stem Sample.

Results of seasonal study - 1955 (Lucerne).

All results as percentages of the dry weight (except moisture).

N.D. = Not determined.

Table 6.

	1L	1S	2L	2S	4L	4S	4F	
Ash content	8.74	10.12	8.55	5.85	9.94	3.81	6.40	
Ether extract	3.91	2.05	-	-	-	-	-	
	5L	5S	5Seed	6L	6S	8L	8S	10S
Ash content	10.49	3.39	4.82	11.95	3.01	9.38	2.88	1.93
Ether extract	5.52	2.68	-	-	-	5.27	3.27	-

Table 7. Meteorological Data.

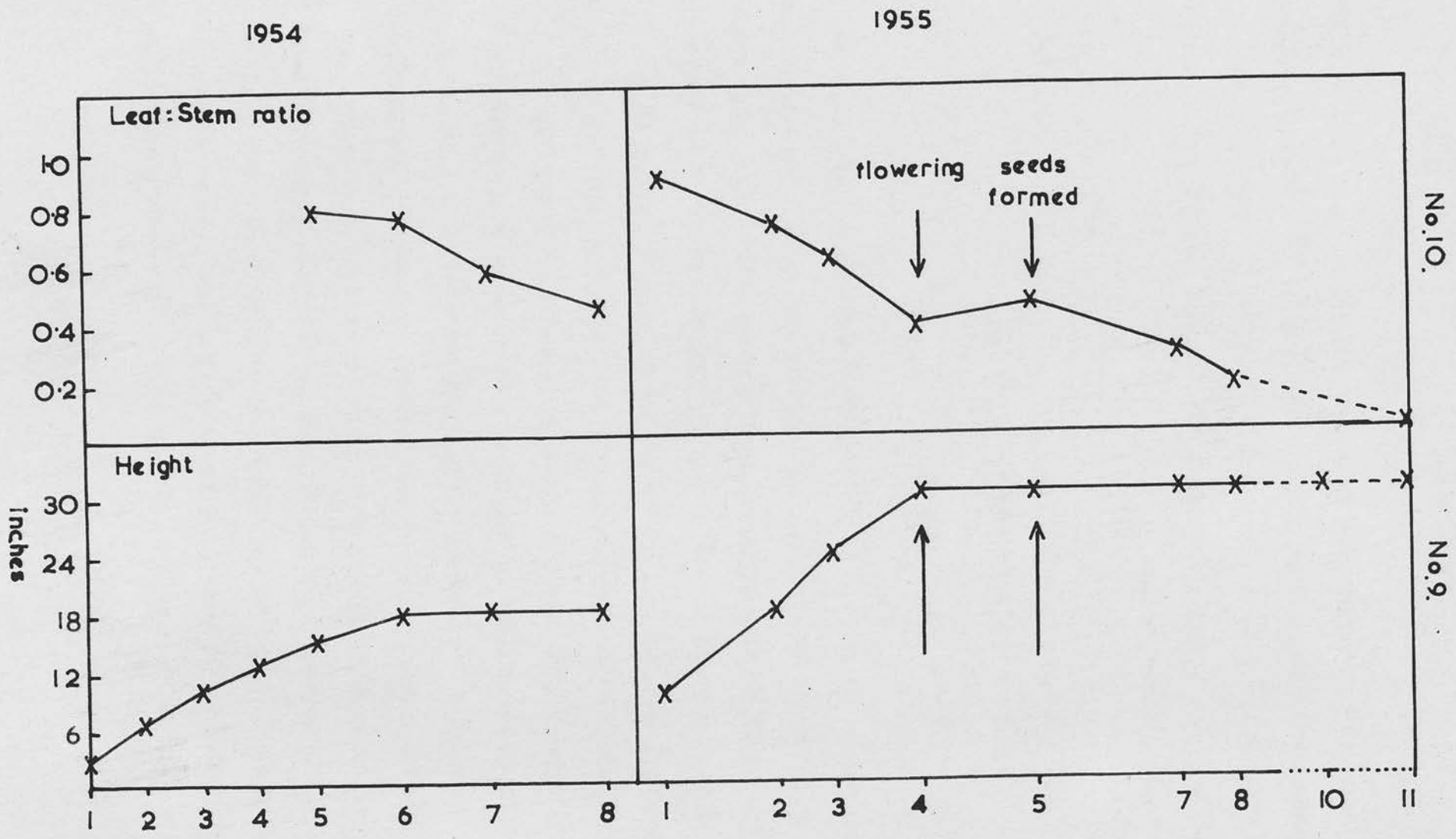
1954	May	June	July	Aug.	Sept.	Oct.	Nov.	
Mean Temperature	55.8	58.5	61.3	60.9	57.4	55.1	47.1	
Rainfall (mm.)	129.9	67.3	52.1	144.3	102.8	167.9	96.6	
1955	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Mean Temperature	54.6	60.8	69.1	68.3	61.5	52.0	49.3	43.0
Rainfall (mm.)	62.2	17.6	54.4	29.2	45.8	43.7	19.7	140.5
1954 Samples	1-2	2-3	3-4	4-5	5-6	6-7	7-8	
Total sun (hours)	44.7	64.7	16.7	44.0	107.7	51.4	52.8	
Number of Days	13	13	13	12	20	20	26	
Daily average	3.7	5.0	1.3	3.7	5.4	2.6	2.0	
1955 Samples	1-2	2-3	3-4	4-5	5-7	7-8	8-10	10-11
Total sun (hours)	228.4	43.6	187.0	172.0	170.7	42.3	113.9	64.9
Number of Days	28	12	20	28	33	13	43	61
Daily average	8.2	3.4	9.4	6.2	5.2	3.3	2.7	1.1

In order to determine how much of the dry matter of the plant was accounted for, the other constituents viz. ash, uronic anhydride, ether-soluble material were estimated in a few of the samples during 1955. The total percentage of the dry matter estimated is shown in the table below.

1L	1S	5S	8S
92.18	88.59	87.83	86.97

These results show that about 90% of the plant material has been accounted for in the analyses. Recoveries of this order have been reported (56) for the analysis of clover samples. The ash, crude protein, uronic anhydride and ether-soluble material were estimated from oven-dried samples and it is possible that this may be responsible for low recoveries found, due to losses during drying.

In any experiment where a small subsample is taken for analysis, the possibility of sampling errors cannot be ignored. This is particularly important in herbage samples where milling or mincing prior to subsampling cannot be carried out. The procedure described was chosen in order that as representative a sample as possible was collected, and that the trends in the percentages of the various constituents would not be masked by variations due to sampling differences alone. It is probable that these errors were more significant than those in the analytical procedure since the


1954
1955
Leaf : Stem ratio
1·0
0·8
0·6
0·4
0·2
flowering
seeds formed
No. 10.
Height
inches
30
24
18
12
6
1 2 3 4 5 6 7 8
1 2 3 4 5 7 8 10 11
No. 9.

conditions in the latter were controlled within narrow limits; the greatest error being that of 5% in the Somogyi estimation.

The growth of the lucerne. In its first year, the lucerne did not reach its maximum height until sixteen weeks after sowing (graph 9). At the time of the first sample (15th July 1954), the lucerne was three inches high, and it grew steadily until 29th September when the height was 18 inches after which no further increase in height was evident. In the second year of growth, the lucerne was about 9 inches high when the first sample was taken (24th May 1955), and constant height (30 inches) was reached two months later (26th July).

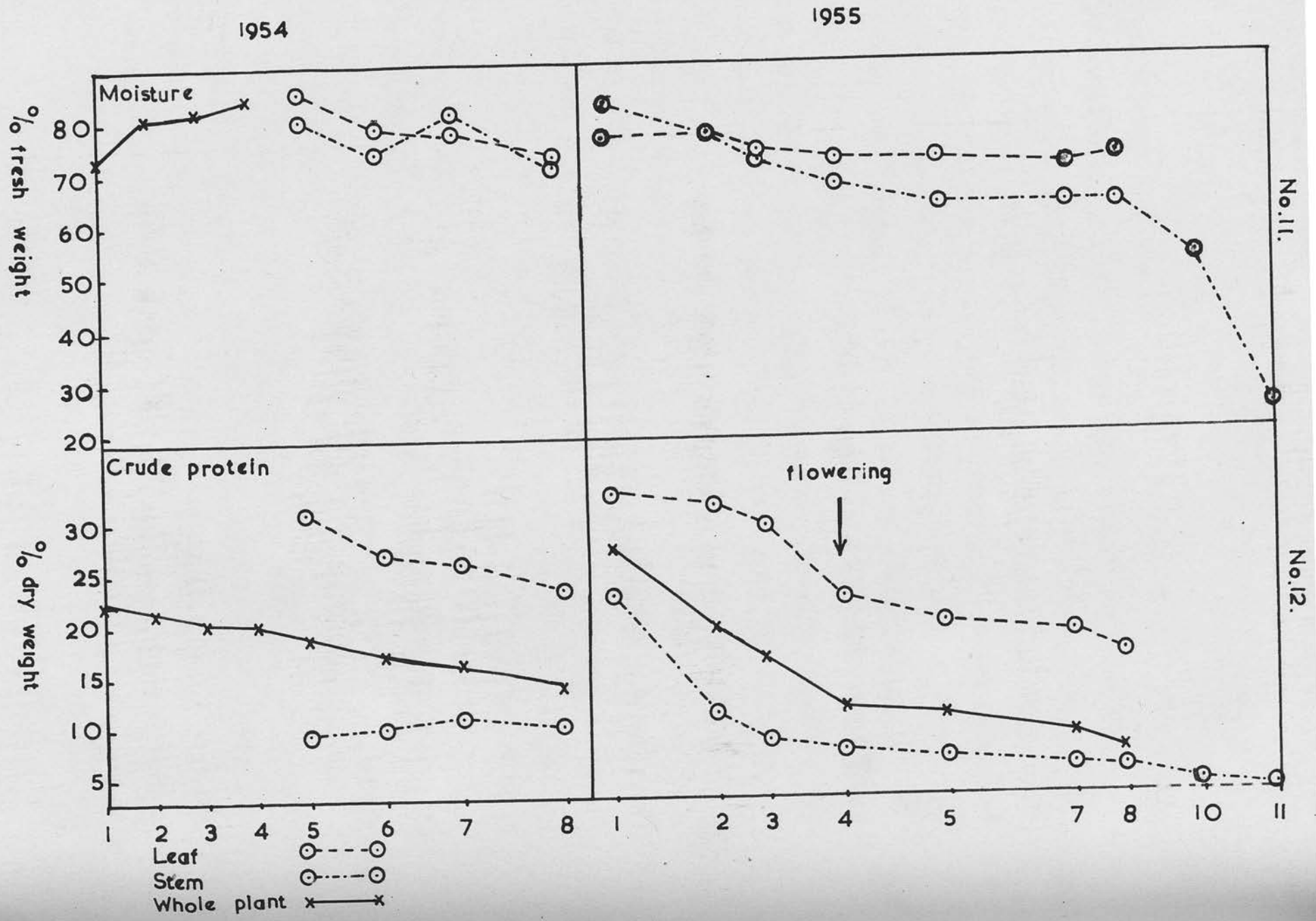
The increase in height of the lucerne was reflected in the decrease of the leaf:stem ratio (graph 10). This ratio was not determined for the very young plant, but during the latter half of the 1954 season, the ratio fell from 0.8 to 0.4, and during the whole of the second year's growth it decreased from 0.9 until all the leaves had withered and fallen by the end of the year. In November, the amount of leaf was insufficient for the collection of an analysis sample; in January no leaves were left on the plant which was completely withered (moisture content 21.4%; graph 11).

The problem arose of explaining the continued steady fall in the leaf:stem ratio during the time the plant was not increasing in height. It is known that increases in the dry weight of grasses occur after they have reached constant height (p. 94) due to thickening of the stem, and this brings about the continued fall in

the ratio. It is probable that this is also the case in lucerne in which the stem becomes very woody. During the 1955 study, the average dry weight of each stem, plus the leaves on it, was determined on a number of samples. These weights were 396 mg. for sample 1, 892 mg. for sample 3, 2070 mg. for sample 5 and 2615 mg. for sample 7, i.e. the leaf + stem weights do show an increase at the end of the season despite the loss of leaves. (In sample 5 the weights of seeds + seed cases was not included.)

Although a few flowers were observed on the first year growth, the crop was not considered to have flowered since not more than one in a hundred of the plants developed flowers. In 1955, the plot had flowered fully by July 26th when a sample of the flowers was taken for analysis. Flowering was followed closely by seed formation and a sample of the seeds (plus their cases) was gathered for analysis on August 24th, by which date the plant had reached constant height.

Two samples of second growth were taken in 1955. For the first aftermath sample, the lucerne was cut before flower formation (sample 2) and the aftermath had flowered and formed seeds by the time it was re-cut (September 7th). The second aftermath sample was from lucerne which had already flowered (sample 4). When the young growth was cut again (October 19th), no flowers or seeds had appeared. Both samples had reached a height of ca. 20 inches during the periods between cutting, the rate of growth being comparable with that at the beginning of the second year.

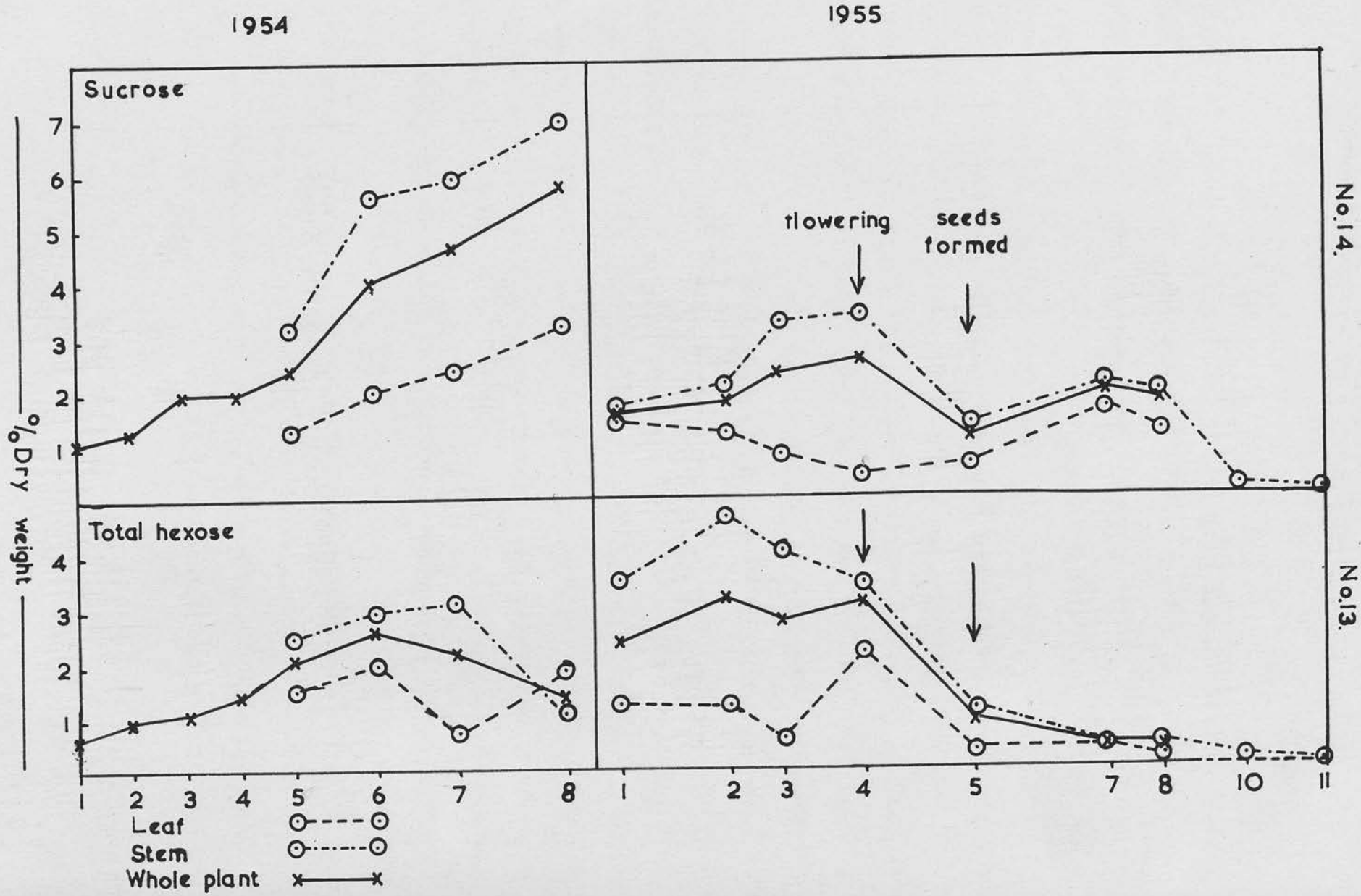

1954
1955
Moisture
% fresh weight
80
70
60
50
40
30
20
No.11.
Crude protein
% dry weight
30
25
20
15
10
5
flowering
No.12.
1 2 3 4 5 6 7 8
1 2 3 4 5 7 8 10 11
Leaf
Stem
Whole plant

Changes observed in non-carbohydrate fractions. The ash content of the lucerne fell during the 1955 season from 9.5% in May to 3.8% in October. The flower and seed (plus cases) were found to contain 6.4% and 4.8% ash respectively.

The slight increase (3.14% to 3.56%) found in the amount of ether-soluble material in 1955 [i.e. fats, pigments (46)] was a reflection of the change in the amount present in the stem (2.05% to 3.27%), the higher content in the leaf (3.91% to 5.27%) having little effect on the total amount because of the low leaf:stem ratio in the later samples.

Lucerne is an important source of nitrogen in farm foods, and the leaves of young lucerne are rich in crude protein, which accounts for about a third of the dry weight. During the 1954 growth, the crude protein fell from 22.5% to 13% of the dry weight of the whole plant. The change was much greater during the following year when, because of the large change in the leaf to stem ratio, the crude protein content fell from 27% to about 7% of the dry matter (graph 12), the change being more rapid before flower formation than after.

Reports in the literature during the past forty years have shown that these trends have been commonly found for the ash (27,54,97,128,146), ether-soluble material (97,146,147) and protein (3,54,128,146,147).


1954
1955
Sucrose
Total hexose
% Dry weight
flowering
seeds formed
No.14.
No.13.
Leaf
Stem
Whole plant

Changes in the carbohydrate constituents. For ease of comparison some of the results have been plotted graphically and these have shown that the pattern of changes in the first year crop differed greatly from that in the second.

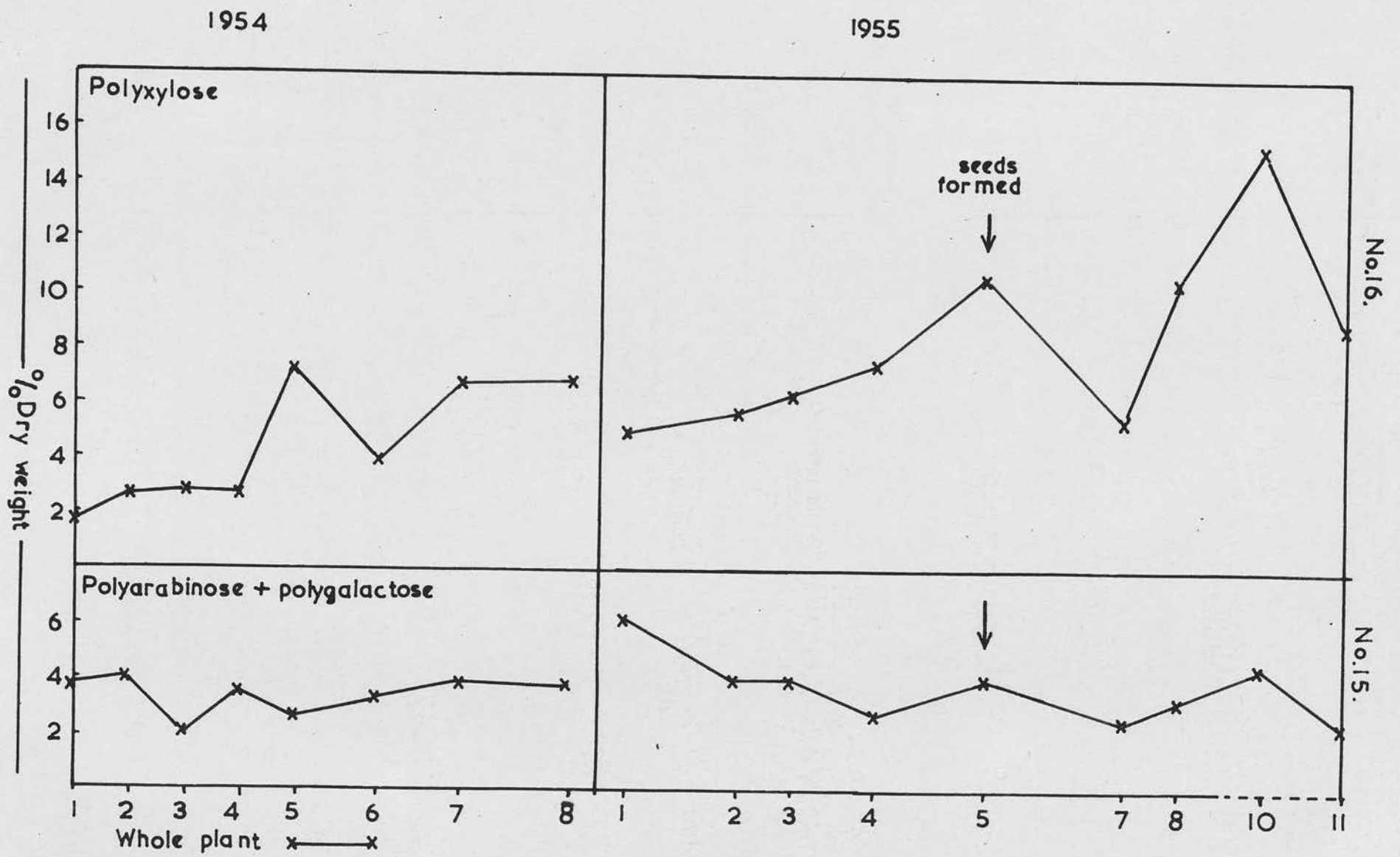
The hexose (glucose plus fructose) and sucrose contents are shown in graphs 13 and 14 respectively.

The amount of oligosaccharides present in both the leaf and the stem was very small, and although slight fluctuations were found during 1954 (around an average of 0.6%), the amount during the following year remained practically unchanged (at 0.25%). From the small amounts present, it is obvious that the oligosaccharide fraction does not form a very important nutritive source of carbohydrate for animals, and cannot be compared with the short chain fructosan fraction found in perennial ryegrass.

Discounting the probable variations due to sampling, no major trends in the amount of polyarabinose plus polygalactose were evident (graph 15).

The amount of mannose-containing polysaccharide was small; the stem contained the larger amount (2% to 3%) and the content for the whole plant thus increased throughout the season (0.65% to 2.70%).

A number of anomalous results have been found for the polyxylose content but a general increase in the percentage was observed (graph 16). The quantity of polyxylose was very small in the young plant (2% of the dry matter) and had risen to about 7% by the end


1954
1955
Polyxylose
Polyarabinose + polygalactose
% Dry weight
seeds formed
No.16.
No.15.
Whole plant
16
14
12
10
8
6
4
2
1
2
3
4
5
6
7
8
10
11

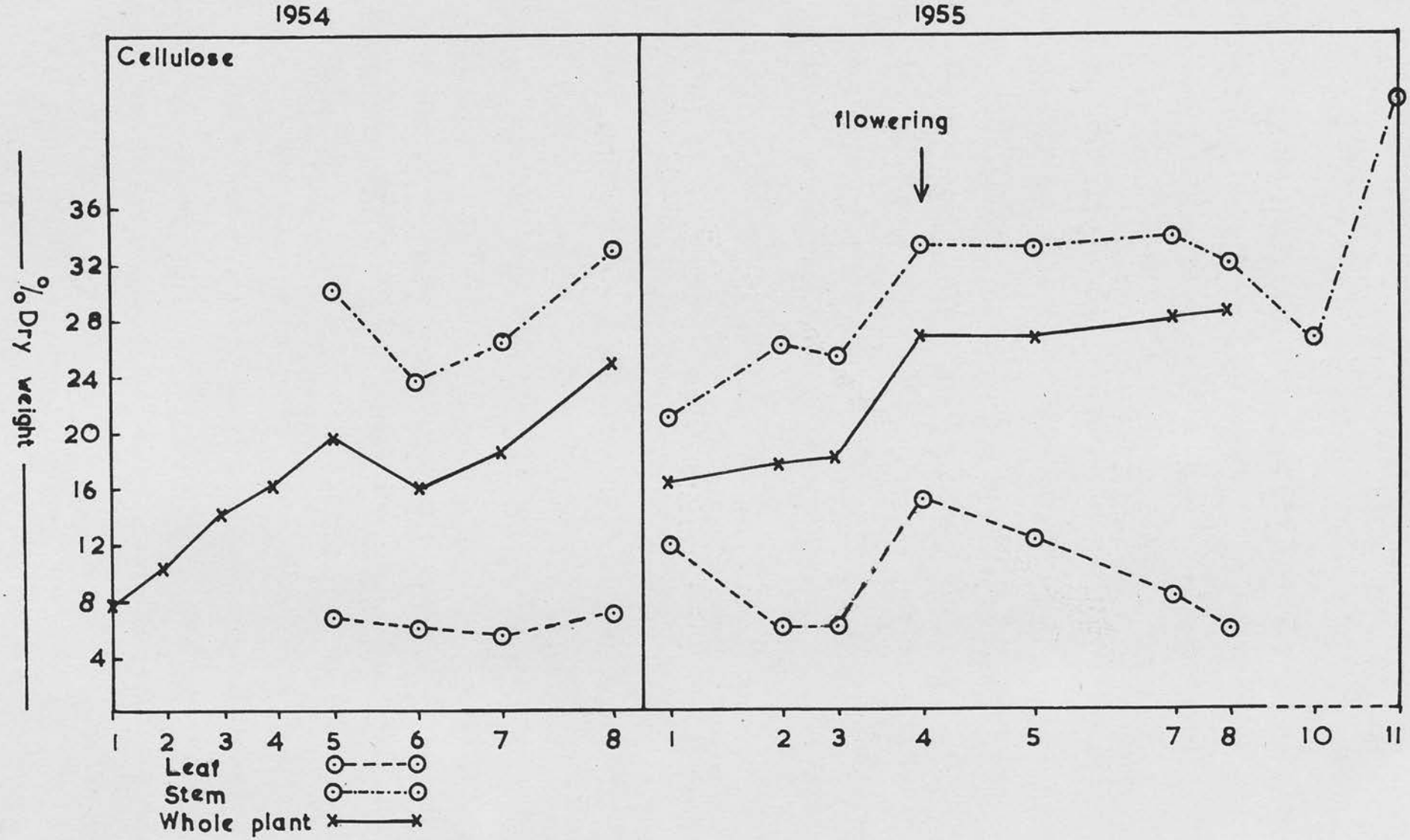
of the first year. In 1955, the young lucerne had a polyxylose content of 5% in the earliest sample and this was found to increase as the plant matured. Considerable variation was found in samples 7 and 10 in 1955; the reason for these fluctuations is not clear.

Little is known about the fate of polysaccharides during the development of the plant, and the experiments carried out for this seasonal study can provide no detailed information about the changes occurring in the individual carbohydrates. It is thought that the plant polysaccharides are being continually renewed, i.e. some monosaccharide units are added to a molecule and others removed. The rate of this interchange may not be constant and will be influenced by the amount of photosynthesis and the plant's requirements for new tissue formation.

The single fluctuation in an otherwise smooth curve for the polyxylose content in 1954 may be due to experimental and/or sampling errors, but the fluctuations occurring during the latter half of 1955 are much greater and it is unlikely that these are due to these two sources alone.

At the time these variations occurred in 1955, the seeds had formed. It is not known, however, whether their development is complete, and it is possible that the changes in the pentosan content may be in some way related to a further development of the seeds.

A second, and possibly more important, cause of changes in carbohydrate content is translocation. Towards the end of the summer the decreasing percentages of carbohydrate in the aerial


1954
1955
Cellulose
flowering
% Dry weight
36
32
28
24
20
16
12
8
4
1 2 3 4 5 6 7 8
1 2 3 4 5 7 8 10 11
Leaf
Stem
Whole plant

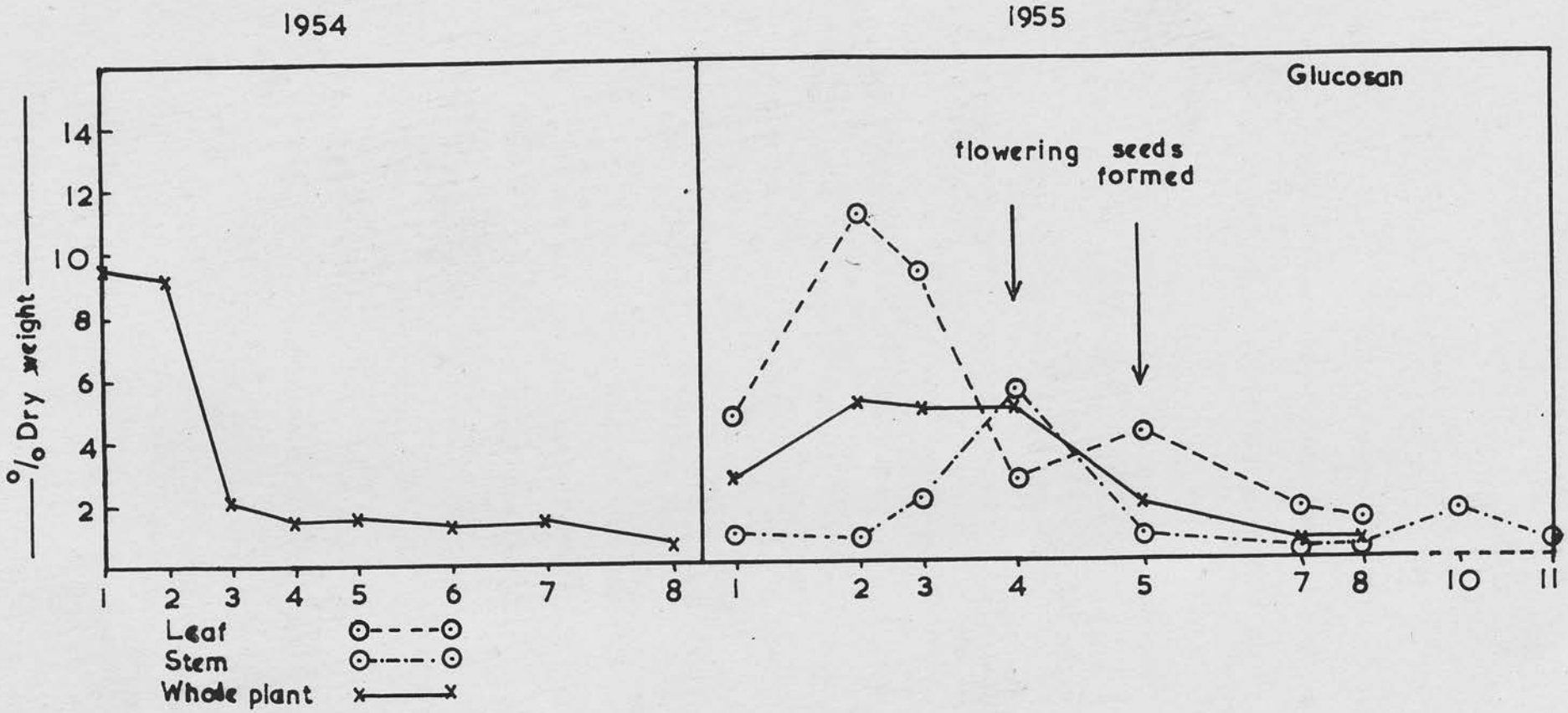
portions of the plant may be due to the lucerne building up, in its roots, reserves for new growth in the Spring. Such storage may involve removal of the stem hemicellulose (e.g. xylan) since the amounts of hexoses, sucrose and oligosaccharides were very low at the time.

Seasonal changes in the amount of the glucose fractions present widely differing pictures in the two seasons. The cellulose results in the first year (graph 17) show a gradual increase in percentage, the content in the leaf, however, remaining constant. This increase was also found in the early part of the subsequent year, but the content did not change much after the plant had reached constant height (at sample 4). The very high value found in January may be due to decreases in the amounts of other constituents e.g. the ethanol-soluble sugars.

No direct estimation of the "starch material" (see p. 151) in lucerne was made. A constituent giving a blue colour with iodine could be extracted with hot water. In the seasonal analyses, the amount of polyglucose removed during the 1N acid pretreatment was used as a comparative indication of the starch content. It must be realised that this value may not represent accurately the amount of starch in lucerne, but it is reasonable to interpret any major changes occurring in the amount of glucose in this extract as variations in the amount of this "starch".

In her study of the seasonal variations of the carbohydrates in

No.18.


1954
1955
Glucosan
flowering
seeds formed
% Dry weight
14
12
10
8
6
4
2
1 2 3 4 5 6 7 8
1 2 3 4 5 7 8 10 11
Leaf
Stem
Whole plant

Subterranean clover, Hardwick (56) found a fraction which she has called "reserve carbohydrate". This was estimated by enzymic hydrolysis under conditions suitable for a starch hydrolysis and determination of the monosaccharides by their reducing power. This term "reserve carbohydrate" [suggested by McIlvaine (87)] denoted a "starch-like" fraction, although because of the difficulty in extracting it with water, it must differ in some respect from the cereal starches.

In the analytical results, therefore, the glucose extracted during the dilute (1N) acid extraction has been called "glucosan" and the amount quoted as a percentage of the dry matter of the lucerne sample. Additional justification for the comparison of "glucosan" and "starch" may be found in the results of the preliminary experiment (p. 67). By 1N acid, the glucose removed was 0.43%, and by the hot water extraction, the percentages were 0.41 and 0.37.

The percentages of glucosan in the lucerne showed quite different trends during the two seasons (graph 18). In 1954, except for the first two samples, the content was low (1% or 2% only) and remained at that level in both the leaf and the stem throughout the entire season. During the next year however a broad maximum was found for the percentage in the whole plant. After mid-August, the percentage fell to the low level found during the previous year. The amounts in the leaf were generally greater than in the stem and during 1955, the leaf value showed a peak (11%) on June 22nd, while the content of the stem reached a maximum (6%) about five weeks later.

Henrici in South Africa (68) did not detect high starch contents any any particular times of the season and found that low moisture levels caused the amount of starch in the leaf to decrease.

Changes in the percentage of "lignin" (i.e. acid-insoluble residue) followed those of the cellulose fairly closely; a steady increase was found during the first year and during the early part of the second with a fall in the rate of increase of percentage after the plant had reached constant height.

The flowers of lucerne did not account for a large portion of the total weight of the plant (only about 10% of the dry weight), the ratio flower:stem being only 0.15. The small purple blooms were very rich in free hexose compared with the leaf and the stem, the glucose and fructose contents being 3.07% and 3.52% respectively, although the sucrose was only 1.67% of the dry matter. The large amount of free hexose resulted in the flowers having a greater percentage of free sugars than ever found in the leaf or stem at any stage of the two seasons' growth. (In this respect they are very like clover flowers which are found to be very sweet to the taste.) The similarity between lucerne and clover had been noted earlier (18) when a decrease in the feeding value of lucerne and red clover had been found to follow the loss of leaves and blossoms.

The percentages of polysaccharides were comparable with those found in the relatively young plants except that the proportion of cellulose was less. Ash, crude protein, uronic anhydride and lignin contents were also of the same order as those found in the less mature whole plant (e.g. sample 1 or 2 in 1955).

The seeds of lucerne are enclosed in helical-shaped cases, the whole accounting for 23% of the dry weight of the plant at the time sampled. On August 24th, a sample of lucerne was separated in leaf, stem and seed (including seed cases). Analysis of the seed plus cases showed that although the 80% ethanol-soluble sugar content was of the same order as those found in the early samples of the plant (Nos.1,2 or 3 in 1955), there was, as in the flowers, a lower cellulose content in the polysaccharide consituents.

Both the flowers and seeds plus cases gave a blue colour when iodine was added to hot water extracts. There was however no large amount of "glucosan", 2.75% in the flower and only 1.18% in the seed portion.

The uronic anhydride content of the seeds was very high (28.43%), greater than found in any other leaf or stem sample analysed (pp.103-104). It is not known whether this is due to an increased content of pectin or to uronic acid residues attached to the polysaccharide constituents. The flowers were only moderately rich in uronic anhydride (11.54%).

The crude protein contents of both parts were high (25%), a value comparable with the leaves of young lucerne.

Interpretation of the analytical results.

In perennial ryegrass, it had been seen that the variations in

carbohydrate composition could not be related to any single factor but were controlled by a number of factors each functioning simultaneously. These factors were found to be (a) rate of growth, (b) stage of growth, and (c) weather conditions.

The young lucerne in 1954 increased steadily in height during its first sixteen weeks' growth (graph 9), and in this period, therefore, there was a steady demand for the photosynthetic products; any increase or decrease in the amount of sunshine would have had a direct effect on the rate at which the hexoses, sucrose, oligosaccharides and "glucosan" accumulated in lucerne. Studying the changes in samples 1 to 6, it was found that the sucrose content (graph 14) increased according to the amount of sunshine received by the plant. The average number of hours of sunshine per day for the periods between the samples are given in table 7. Between samples 1-2 and between 2-3, the average daily sunshine was 3.7 and 5.0 hours respectively. Between samples 3-4, there was a spell of poor weather, only 16.7 hours bright sunshine being recorded on the thirteen days between sampling, and this was reflected in the break in the sucrose curve. Thereafter the average sunshine figure was high, and the sucrose content again rose.

After sample 6, the lucerne did not increase in height and (as seen with the 1955 samples) the growth of the plant continued only by an increase in stem weight. Despite this apparent reduction in the amount of material required for new plant tissue, the rate at which the sucrose was stored was slightly lower than in the early

part of the season due probably to the reduced amount of sunshine received by the plant, and also to the requirements for translocation to the roots.

The lucerne in 1955 grew more rapidly, attained a greater height early in the season and passed through the various stages of development viz. flower and seed formation and the loss of its leaves at the end of the season.

A steady increase in the sucrose content was not found, although there had been plenty sunshine throughout the whole summer. It is probable, therefore, that the products of photosynthesis in the more mature plant do not accumulate as sucrose molecules but appear as the cell-wall materials, the reserve polysaccharide and the protein required in the growth of the plant.

In ryegrass, it appeared that the fructosan and oligosaccharides represented the balance of the photosynthesised carbohydrate which was not required for the growth of new tissue and the metabolism of the plant. In lucerne, the roles of fructosan and oligosaccharides are taken by the "glucosan" and to some extent by the sucrose.

The amount of "glucosan" (graph 18) found in the plant in 1954 was small except in the first two samples. One possibility for these high values is that in these samples of the very young leafy lucerne, the plant was able to photosynthesise more than enough carbohydrate required for the formation of new tissue (e.g. for stem growth) and this carbohydrate accumulated as "glucosan".

The two values however are much higher than might be expected

by comparison with the subsequent samples, and some doubt must arise that they are not due to this starch material. Francois, who has studied the degree of polymerisation of the cellulose in lucerne, obtained results (see p.17) which indicated the presence of shorter chain length molecules (ca. 300 glucose units) in the leaf cellulose than in the stem. The existence of such short chain cellulose molecules occurring in a living plant has not been proved, and molecules of this size may have arisen by degradation of the natural cellulose chains during isolation. It is possible that in the non-lignified tissue of the young lucerne (in samples 1 and 2) the normal sulphuric acid is able to attack the cellulose more readily than a mature lignified tissue (which is encrusted with hemicellulose and requires 72% sulphuric acid). By dissolving fragments of the molecule, it will give rise to increased amounts of glucose in the first acid extraction of the sample.

During 1954, the plant increased in height, more and thicker stem was produced, and towards the end of the summer when active growth is slowing there will be demand for carbohydrate for storage in the roots for the growth in the next Spring. It is suggested, therefore, that the plant was unable to accumulate any reserve of carbohydrate in the above ground portions, giving the low "glucosan" contents found in samples 3 to 8 in the first season.

The picture in the following year was quite different. The lucerne had been cut back at the end of November 1954 and the new

crop began to grow in the Spring. At the time of the first two samples the weather was very sunny (an average sunshine of 8.15 hours daily), the amount of leaf on the plant was high and thus the conditions for photosynthesis were good. This was confirmed by the increasing percentages of mono-, di-, oligosaccharides and "glucosan". The good weather continued after this, but the total percentage of these four components did not increase, and because there was no rapid increase in the percentage of the polysaccharide components at this period, the halt in the accumulation of water-soluble carbohydrates was unexpected.

It was, however, at about this stage that the growing point changed from the vegetative to the floral state, i.e. when the flowers began to develop. At the time sample 3 was taken (July 5th), it was noted that the lucerne was just about to flower, the flower-heads had appeared although they had not burst into bloom, and by sample 4 (July 26th), the crop was fully flowered. From the end of June, therefore, until about this date, it is possible that energy demands, by the plant, reduced the proportion of photosynthesised material which the lucerne could store. It appeared that flower formation upset the metabolism of the plant by making increased demands on the carbohydrate.

Changes in nutritive value at this stage of growth have been reported. In 1927 (97) it was recorded that the "starch value" of lucerne hay cut before blooming was higher than that cut after. For silage-making a high water-soluble sugar content is desirable,

and for lucerne silage, cutting when between 10% and 25% of the flowers had appeared was recommended (55). It was at the "10% flower" stage that lucerne was found to have the highest nutritive value (126).

The pattern of the changes in the plant carbohydrates during growth shows that the photosynthesised sugar in excess of the plant's immediate requirements for growth occurred as starch in the leaves (samples 1 and 2, 1955). After this date, the additional demands made by the plant for flower development reduced the amount of starch which was accumulated and the percentage fell between samples 2 and 4. By this time, the lucerne had flowered and seed formation would be in its early stages. Thus there would be a demand for carbohydrate to form the seeds (shown by the fall in percentage of hexoses, sucrose and "glucosan" in sample 4). The stems which showed a peak for "glucosan" content at this date apparently contained the carbohydrate to be used for seed development. The mechanism of this transfer of carbohydrate from the leaf to the seed is not known, but from the results of these experiments it appeared that in addition to the transfer of the water-soluble sucrose through the stem, the "glucosan" also entered into the scheme. It is possible that since there is a greater amount of glucosan in the stem than normal, the glucose residues are transferred from one molecule to another until they arrive in the seed where they can be used for its development. This would explain the transfer of glucose units in a form in which they could not be extracted with

80% ethanol although no evidence of such a system has ever been reported.

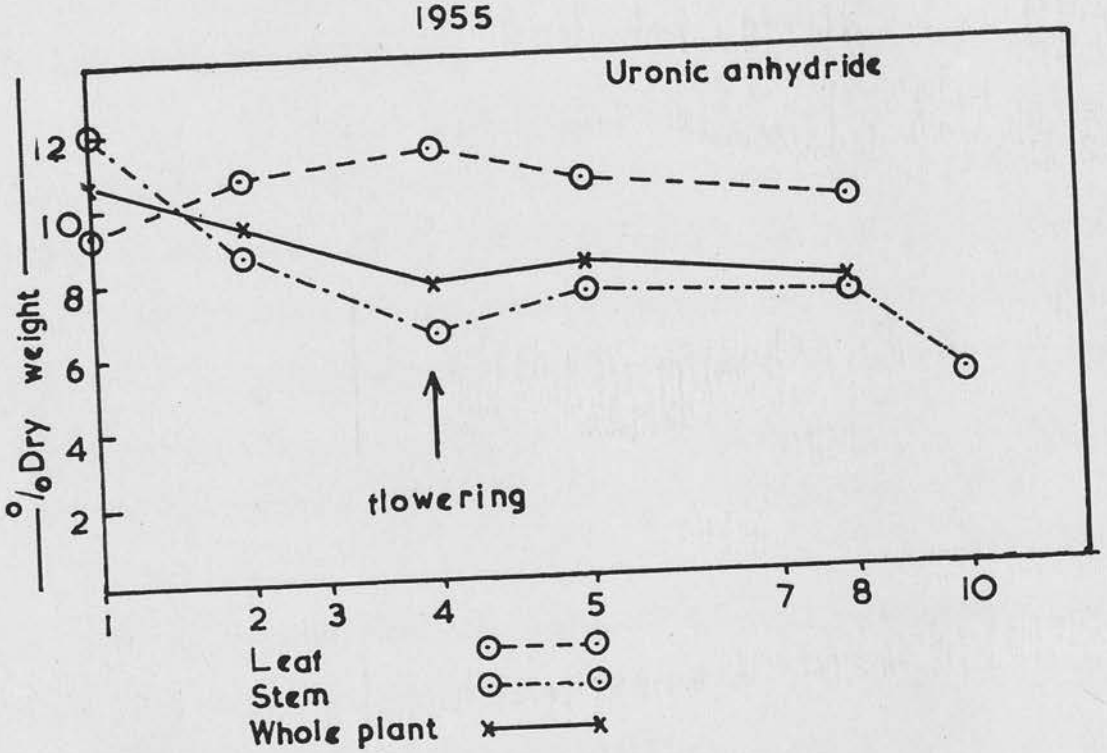
The possibility that the hemicellulose polysaccharides also take part in this transfer of carbohydrate has already been noted (p. 111) and the variations in amount of both hemicellulose and "glucosan" in the stems at the time of seed development indicate that these polysaccharides do play a part in the plant's metabolism and are not completely isolated as cell wall material.

By the time the seeds had fully formed, the plant had reached constant height, and therefore no rapid growth was taking place. There appeared to be no excessive demands for carbohydrate and the leaf should contain a greater reserve of carbohydrate. This was found (sample 5), although because of the decreasing leaf to stem ratio, this did not appear as an increase in percentage in the whole plant.

Considering the changes observed in the amounts of cell wall materials, the main trend was the increasing production of cellulose and lignin. The picture was slightly different during the two years. In 1954 a steady increase was evident in the amount of cellulose, but during the subsequent year the percentage remained constant after July 26th.

Hardwick in her study of clover during its third season's growth found (56) that the cellulose and lignin contents showed graphs of similar shape. In clover, the percentage of cellulose and lignin remained constant over the early part of the season until

No.19.


1955
Uronic anhydride
%Dry weight
12
10
8
6
4
2
flowering
1
2
3
4
5
7
8
10
Leaf
Stem
Whole plant

the plant started to wilt, when both increased rapidly. This was in contrast with the gradual increases found in lucerne during growth before becoming constant towards the end of the season.

During the process of lignification, the content of pectic materials in plants usually decreases, and in hard tissues such as wood, they may only represent a small proportion of the plant substance (75). The young lucerne in 1955 was found to have a uronic anhydride content of about 11% and as the material aged the percentage fell (graph 19). As it has been shown (p.153) that the major part of this uronic acid content was present as pectic material which could be extracted with ammonium oxalate, this decrease was in agreement with the evidence of lower pectic contents in woody plants. The fall, more rapid in the early part of the season than in the later, may not reflect a fall in absolute amount.

The aftermath growth. Comparison of the two aftermath samples has been interesting in that their growth behaviour was different. The first sample, cut before flowering, gave an aftermath which produced flowers and seeds, whilst the second sample, cut at the time of flowering, remained in the vegetative state.

Both samples reached the same height (ca. 20 inches) but the later sample had a much higher leaf:stem ratio (0.67 cf. 0.41). The ethanol-soluble sugar content was also very slightly higher than in the earlier but the "glucosan" value was lower. This meant that despite the difference in growth, the amount of water-soluble

carbohydrate was similar in both samples (6.9% cf. 6.5% dry matter). The same was not true of the polysaccharide fractions in which the results show that less stem production in the later sample* was reflected in the lower contents of all the polysaccharide components and "lignin".

* Average weight of 1 stem plus the leaves on it was 730 mg. cf. 1390mg.

The wilting of lucerne.

The moisture content of the lucerne showed a gradual decrease with plant maturity (see graph 11). In the first year, it increased slightly as the very young plant developed, but in the established crop, the value fell from just less than 80% in the early part of 1955, to 51% (December) and 21% (January) in the stems. (The last two samples were not taken at any date when lucerne would normally be used in agricultural practice. At the time the January sample was taken, the above ground portion of the plant appeared dead.)

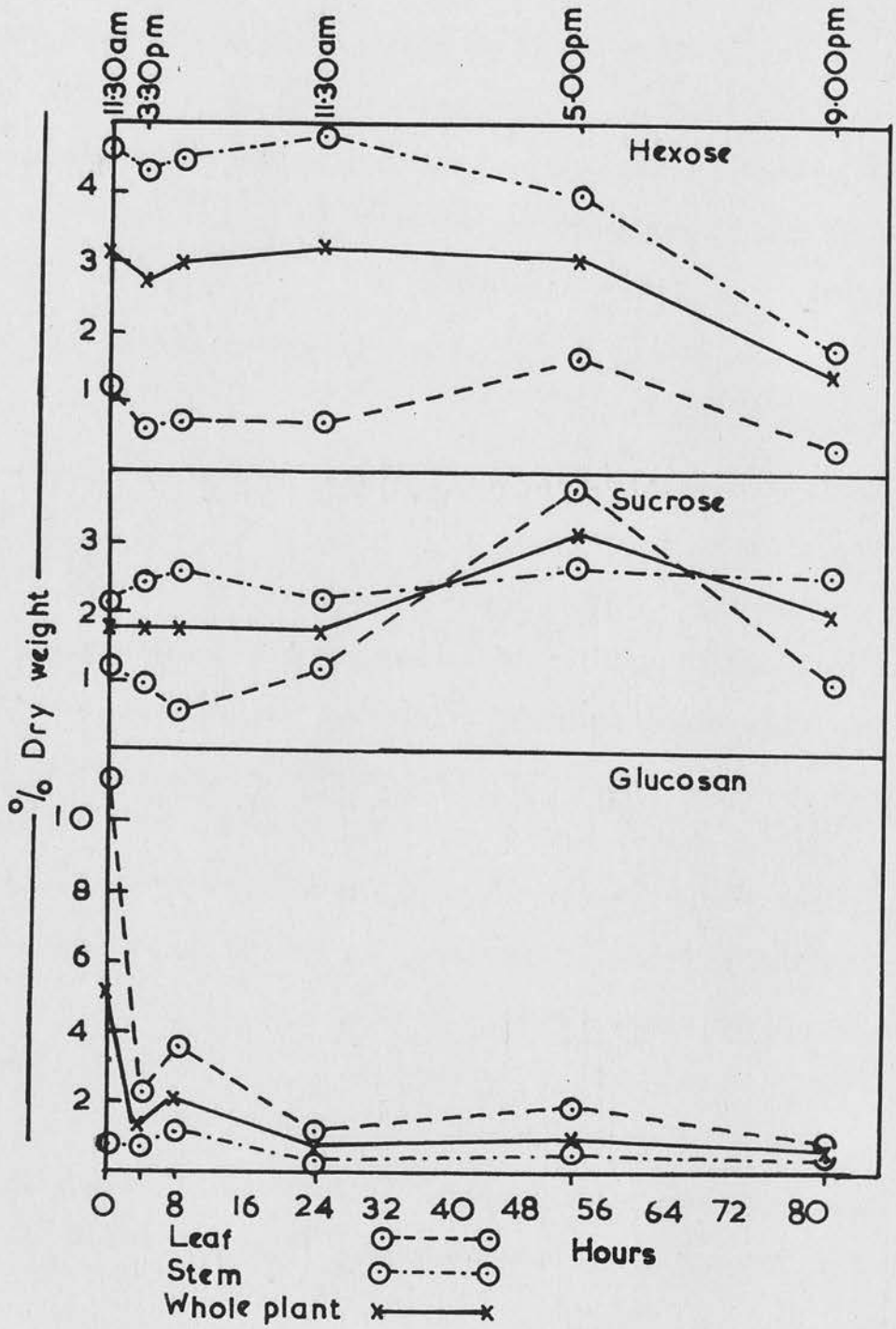
During the wilting of lucerne, it had been observed (73) that the leaf aided moisture loss. Photographs taken of the leaves during wilting have shown a re-opening of the stomata two hours after cutting. The moisture loss was thus much more rapid from the leaves than from the stems, where the internal water loss was by evaporation through the stem epidermis (76) and was hastened by crushing.

A wilting trial, carried out in the laboratory, showed that the leaf and stem dried at different rates (graph 23). During the

No.20.

No.21.

No.22.



experiment (82 hours), the moisture content of the leaf fell from an initial 75.6% to 14.3% while that of the stem changed from 75.7% to 35.4%.

The results (table 8) show that very little change occurred in the hexose (i.e. glucose + fructose) content of the lucerne during the first 48 hours of the experiment (graph 20). The amount in the stem was greater than in the leaf and decreased after 24 hours. In the leaf, however, a maximum hexose content was noted after 53½ hours, at 5.00 p.m. It is impossible to decide from this experiment whether this increase is due to the plant's metabolism being upset or whether it is due to a diurnal effect even although the plant had been cut.

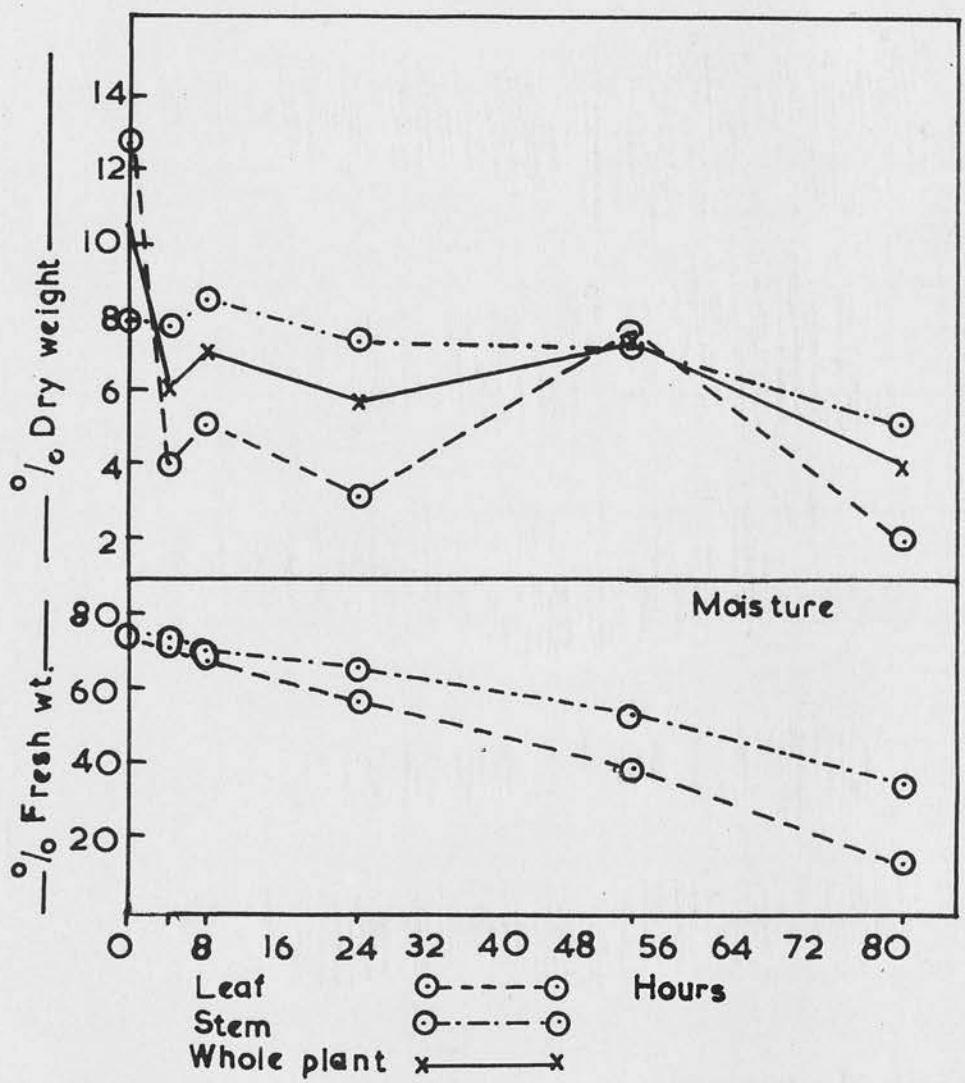
The amount of sucrose (graph 21) in the stem remained practically unchanged (2.11% to 2.55%). In the leaf, however, after an initial fall (1.20% to 0.55%) between 11.30 a.m. and 7.30 p.m., the amount had risen to 1.15% after 24 hours. This suggests a diurnal variation, and the large amount (3.82%) present after 53½ hours appears to support such a suggestion since it had been noted that the sucrose maximum in growing perennial ryegrass (135) and lucerne(67) had been found to occur in the afternoon or early evening. The decrease after 53½ hours was comparable with Henrici's observations (67), that the sucrose content increased temporarily in wilting lucerne; the increase lasted for two days, and then the amount fell rapidly.

Although the identity of the "glucosan" fraction as starch was not certain, it showed some interesting features (graph 22).

Table 8. Wilting experiment.

	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem
Sample number	1	2	3	4	5	6	7	8	9	10	11	12
Date 1955	22.6	22.6	22.6	22.6	22.6	22.6	23.6	23.6	24.6	24.6	25.6	25.6
Time	11.30 a.m.	11.30 a.m.	3.30 p.m.	3.30 p.m.	7.30 p.m.	7.30 p.m.	11.30 a.m.	11.30 a.m.	5.00 p.m.	5.00 p.m.	9.30 p.m.	9.30 p.m.
Hours wilted	0	0	4	4	8	8	24	24	53½	53½	82	82
% moisture	75.61	75.71	71.65	73.25	68.02	71.45	57.19	66.53	39.25	54.28	14.29	35.42
L/S ratio	0.73 : 1		0.70 : 1		0.67 : 1		0.65 : 1		0.62 : 1		0.59 : 1	
Glucose	0.57	2.60	0.24	2.58	0.28	2.38	0.39	2.62	0.93	2.15	0.26	1.07
Fructose	0.62	2.03	0.35	1.71	0.38	2.15	0.31	2.21	0.73	1.85	0.11	0.75
Sucrose	1.21	2.13	0.94	2.37	0.55	2.58	1.15	2.17	3.82	2.67	1.02	2.52
Oligosacchs.	0.13	0.40	0.19	0.33	0.43	0.27	0.13	0.07	0.25	0.09	0.09	0.07
"Glucosan"	11.16	0.78	2.19	0.67	3.46	1.16	1.17	0.29	1.87	0.44	0.52	0.74

No.24.


% Dry weight
14
12
10
8
6
4
2
Moisture
% Fresh wt.
80
60
40
20
0
8
16
24
32
40
48
56
64
72
80
Hours
Leaf
Stem
Whole plant

No.23.

Initially the leaf was found to contain a large quantity of glucosan (11.16%), and the amount, after an initial decrease, varied during the experiment (1% to 2%), and it appeared that subsequent changes were due, in part at least, to diurnal effects, occurring as they do at 7.30 p.m. and 5.00 p.m. The "glucosan" content of the stem decreased only very slightly throughout the experiment.

It was not likely that large changes would occur in the quantities of hemicelluloses and cellulose during the 82 hours in which the lucerne was studied. From graph 24 (the total ethanol-soluble sugars plus "glucosan"), it is seen that the carbohydrate content decreased as wilting progressed. After an initial rapid loss of "starch" [also found by Wilson (145)] and of some hexose, the amount showed a gradual decrease interrupted by diurnal effects viz. increased amounts of sucrose and "starch". Towards the end of the experiment, the plant seemed unable to respond to diurnal influences and the amount of carbohydrate fell as the plant died, i.e. after the moisture content of the plant fell below 40% to 50%, the plant enzymes seemed unable to function normally.

These results revealed the same overall trends found by Henrici (67), the leaves wilting more rapidly than the stems. She claimed that the loss of photosynthetic ability in the leaves was to some extent balanced by the stem's action as an assimilating organ, although there was nevertheless a loss of starch during wilting.

PART 2. EXPERIMENTAL

Qualitative work on the carbohydrates of lucerne.

Experimental.

For the study of the actual carbohydrates present in lucerne, a large sample of an established crop of lucerne (du Puits variety) was gathered in late October 1953. The plant (2½ to 3 feet high at the time of cutting) was placed in a large oven at just above 100°C. within ½ hour of being cut. After drying, the plant was milled through a fine mesh in a hammer mill. This was the material used in the examination of the oligosaccharides, polysaccharides and uronic acids present in lucerne.

For the preparation of the starch fraction (p. 151), a large sample of lucerne, gathered on May 25th, 1956, was separated into leaf and stem. The leaves were immersed in alcohol within 1 hour of cutting.

The oligosaccharides in lucerne.

N.B. All evaporations (unless stated) were carried out at 30-35°C. under reduced pressure.

The alcohol was evaporated from the 80% ethanol extracts of ca. 500 gm. lucerne, and the aqueous solution was concentrated to 100 cc. The solution was heated to 95°C. on a boiling water bath and clarified with cadmium sulphate (20 cc.; 0.36N) and barium hydroxide solution (equimolecular volume). The insoluble material was filtered off through a pad of supercel and washed with water. The combined filtrate and washings were evaporated to 150 cc. and

electrodialysed to remove the remaining salts.

Charcoal (60 gm.) and supercel (60 gm.) were mixed to a slurry with water and this formed a column (20 cm. x 3.5 cm. diameter after settling) which was washed with 3 litres of distilled water (142).

The solution containing the sugars was allowed to pass through the column and the monosaccharides were eluted with 2 litres of water. At this stage, 130 cc. of eluate gave no stain after it was concentrated and spotted onto a filter paper sprayed with aniline oxalate solution. The oligosaccharides were eluted with increasing concentrations of aqueous ethanol, A - 5% (2 litres), B - 10% (2½ l.), C - 15% (2 l.), D - 30% (4 l.), E - 40% (5½ l.). Each of these five fractions was divided, during collection, into a number of smaller fractions which were evaporated and made up to 5 cc. (A_1 A_2 A_3, B_1 B_2 etc.).

Paper chromatographic separation of oligosaccharides. In identifying oligosaccharides by their paper chromatographic behaviour, their slow rate of travel is a disadvantage. Prolonged irrigation on Whatman No.1 chromatography paper led to large spots (due to diffusion) in most solvents. For this reason a thicker paper (Whatman No.3) was selected. The solvent front travelled more slowly on this paper but it was found that the sugar spots were more compact.

The following solvents were tried,

ethyl acetate-pyridine-water (10-4-3 v/v) - solvent iv,

n-butanol-acetic acid-water (4-1-5 v/v) - solvent v,

n-amylalcohol-pyridine-water (7-7-6 v/v) - solvent vi.

Solvent iv was the only one which gave adequate separations into di-, tri- and larger oligosaccharides without very long irrigation times being required.

The technique of multiple development (i.e. irrigation of a chromatogram more than once) was used to separate mixtures of oligosaccharides which travel at approximately the same rates. In order that no fast-running constituent of a mixture was lost during a multiple development separation, ascending solvent chromatography was used in many cases i.e. the solvent was allowed to travel up the paper from a dish in the foot of the tank. Solvent iv required ca. 18 hours to travel up a paper (50 cm., Whatman No.3). When the solvent front had reached the top edge of the paper, the chromatogram was removed from the tank, air-dried and irrigated again.

Examination of fractions from column. From the concentrated fractions (5 cc.), aliquots (1 cc.) were taken, evaporated to a very small volume (0.1 cc.) and spotted onto chromatograms (Whatman No.3) irrigated with solvent iv, overnight at 22.5±1°C. in tanks contained in a thermostatically controlled box.

The papers showed (aniline oxalate spray) that A_1, A_2 and A_3

contained sugars travelling at approximately the same rate as disaccharides (e.g. maltose) but also a mixture of other sugars some running slower and some faster than this.

The fractions $B_{1,2,3}$ and $C_{1,2,3}$ contained a mixture of sugars of different molecular weights. The later fractions (D and E) contained mainly monosaccharides in addition to traces of disaccharides.

Because of the poor separation, the remaining portions of $A_{1,2,3}$, $B_{1,2,3}$ and $C_{1,2,3}$ were combined to give one solution and re-fractionated on a second column which had been pre-treated with stearic acid (4).

Equal weights (30 gm.) of charcoal and supercel were mixed to a slurry and allowed to stand for 1 hour. The mixture was filtered through a pad of glass wool (to separate the very fine particles of supercel) and oven-dried at 80°C. (6 hours). The dry mixture (50 gm. portion) was mixed to a slurry with 95% ethanol and poured into a column (17 x 2 cm. diameter after settling). A stearic acid solution (1 litre; 1% stearic acid in 95% ethanol) was run through the column. The acid-treated material was then blown out of the glass tube onto a Buchner funnel and washed with ethanol (50%; 400 cc. followed by 20%; 400 cc.). The charcoal-supercel mixture was made into a slurry with 10% ethanol and re-introduced into the glass column and washed with water (500 cc.).

The fractionation was carried out in the constant temperature room (18-20°C.). The solution was applied to the column and the

oligosaccharides were separated with aqueous ethanol by the technique of gradient elution (4). Fractions (2 - 2½ ml.) were collected every 32 minutes in tubes held in a rotating rack operated by a clockwork mechanism. Every tenth tube was analysed on chromatograms (irrigated with solvent iv). From the results of these chromatograms, the contents of the tubes were combined to give the following fractions.

Fraction	Tube numbers	Sugars present in the fractions
1	1-25	No sugars present
2	26-55	Monosaccharides - glucose, fructose, pentose (xylose ?)
3	56-125	Monosaccharides - glucose only
4	126-175	Glucose + slow running sugar just faster than raffinose
5	176-240	Glucose, small amount of sugar at about the rate of sucrose
		Sugar at about the rate of maltose
		Sugar slightly faster than raffinose
		Fast-moving sugar, much faster than monosaccharides
6	241-385	Traces of glucose and a disaccharide
		Sugar running at about the rate of raffinose
		Trace of fast-moving sugar found in 5
7	386-500	Traces of glucose
		Sugar running slightly slower than raffinose

From these fractions 1, 2 and 3 were discarded; 4, 5, 6 and 7 were retained for further examination.

Enzymic hydrolysis of oligosaccharides on chromatograms. The presence of fructose units in fractions 5 and 6 was shown by spotting a small amount of the fractions onto a filter paper and spraying with urea oxalate.

In order to determine the conditions required for the enzymic hydrolysis of sucrose links, chromatograms (Whatman No.3 paper) were prepared containing the following spots.

Paper 1. (4 spots). Sucrose, raffinose, sucrose + invertase (superimposed) and raffinose + invertase.

Paper 2. (4 spots). Stachyose, fructose, stachyose + invertase, fructose + invertase.

The invertase was a commercial preparation.

The papers were hung for 60 minutes in a glass tank containing water vapour at 22.5±1°C. After the hour, the papers were removed, air-dried to remove excess water and irrigated twice (with solvent iv) by ascending solvent chromatography. The chromatograms were sprayed with aniline oxalate. The following results were obtained.

From the sucrose + invertase spot - only traces remained unhydrolysed,

from the raffinose + invertase spot - evidence of a faster-moving product (i.e. melibiose) and fructose,

from the stachyose + invertase spot - fructose was produced but the rate of travel of the trisaccharide was only very slightly faster than the parent tetrasaccharide, and

from the fructose + invertase spot - no evidence of synthetic action to produce oligosaccharides.

Separation of the oligosaccharides in the fractions obtained from the stearic acid treated column.

It is probable that the preliminary examination of the fractions did not reveal all the constituents of the fractions and further chromatographic examinations were required. To examine the results of several developments, the following test was made using fraction 5. Six irrigations (ascending) were carried out with solvent iv on a strip (50 x 3 cm.) to which a small amount of the fraction had been applied. The paper was sprayed with aniline oxalate and a total of twelve separate spots were detected, some only in trace quantities (detectable under ultra violet light).

The fractions 4, 5, 6 and 7 were spotted onto strips of Whatman No.3 paper (50 x 13 cm) prepared as quantitative chromatograms (p. 35). These papers were irrigated six times using ascending liquid flow (solvent iv). The papers were air-dried, and the side strips were cut off and sprayed with aniline oxalate solution. Fraction 4 gave sugar spots only 3 of which were in quantity sufficient for further analysis.
Fraction 5 gave 7 spots, fraction 6 also 7 spots, and fraction 7 6 spots. [Fraction 5 did not give such a good separation as obtained previously on a narrow strip.]

The 23 fractions obtained were washed off the separate sections of the strips by exhaustive extraction in a flask with water on a boiling water bath. The extracts were evaporated and examined by paper chromatography.

Fraction 4.

4a. (insufficient for isolation) remained at starting line.

4b. A slow running spot which travelled at the same rate as stachyose in solvent iv, i.e. probably a tetrasaccharide. It was unchanged by invertase on the chromatogram (no fructose was detected) and therefore did not contain a sucrose linkage. After hydrolysis with 0.5N H_2SO_4 for 4 hours on a boiling water bath, only glucose was detected on a chromatogram (solvent ii) of the hydrolysate.

4c. This material travelled very slightly faster than raffinose (in solvent iv) and was unchanged by invertase. Acid hydrolysis (as 4b) showed galactose and glucose the only sugars present. The ratio of these sugars in the hydrolysate, determined after their separation on a paper (in solvent ii) and estimation, was found to be 0.93:1 respectively.

4d. The substance ran slightly slower than a sample of maltose but in front of raffinose on the chromatogram. It did not run at a rate identical to sucrose or cellobiose and after acid hydrolysis (as 4b), galactose and glucose were shown to be present in addition to traces of xylose and arabinose i.e. the fraction was probably a mixture of at least two oligosaccharides, each containing more than one sugar residue.

Fraction 5.

5a. This contained two very slow-moving spots, one slower than raffinose and the other at the rate of that sugar. After invertase

hydrolysis of the fraction on a chromatogram, fructose was detected and a spot which ran at the rate of melibiose. Acid hydrolysis showed the presence of galactose, glucose and fructose.
The two spots were separated on a strip of Whatman 3MM paper, irrigated three times (descending solvent) with solvent iv. The two sugar zones were eluted (by suspending over boiling water) and hydrolysed with acid (as 4b). The ratio of sugars was estimated:
5aA - (the faster of the two spots) contained galactose:glucose: fructose 0.92:1:0.62 (by weight). Correcting for 28% loss of fructose on hydrolysis, the ratio became 0.92:1:079.
5aB. (the slower spot) contained the same three sugars in the ratio 1.70:1:0.70 (corrected).
5b. The sugar ran very slightly faster than raffinose (at the rate of melibiose). After acid hydrolysis (as 4b) only galactose and glucose were detected on a chromatogram in approximately equal amounts (estimated by visual examination of the stains produced with aniline oxalate).
5c. Two spots (A and B) were present, one travelling (solvent iv) at about the rate of maltose and the other slightly faster. Both gave glucose on acid hydrolysis (as 4b) and the faster of the two also gave traces of xylose.
5d. This was one single spot which moved at the rate of sucrose (solvent iv) and on invertase hydrolysis gave glucose and fructose.
5e, 5f, 5g. The amounts of these fractions were very small and due possibly to incomplete removal from the paper, there was insufficient

for further detailed analyses. 5e and 5f travelled about the same rate as monosaccharides (in solvent iv) while 5g was a very fast-moving material which travelled far in front of 5e and 5f.

Fraction 6. (only 3 fractions collected in quantity sufficient for analysis).

6a. The fraction contained two slow-running oligosaccharides which travelled at the same rate on a paper chromatogram (solvent iv) as raffinose and stachyose (6aR and 6aS respectively).

6aR gave on acid hydrolysis (as 4b) glucose, galactose and fructose in approximately equal quantities. Quantitative estimation after separation of the sugars in the hydrolysate on a chromatogram (irrigated with solvent ii) showed that the ratio was 1:0.98:0.60 (corrected). After enzymic hydrolysis with invertase, fructose and a disaccharide which travelled at the same rate as melibiose (solvent iv) were obtained.

6aS. Acid hydrolysis and chromatographic examination of the hydrolysate (solvent ii) showed that glucose, galactose and fructose were present. The ratio was found to be 1:2.20:0.73 (corrected). Enzymic hydrolysis with invertase yielded fructose indicating the presence of a sucrose linkage and a very slow-running oligosaccharide. The galactose content was higher than the expected value (= 2) from stachyose and there may therefore be a higher homologue also present.

6b. Multiple development (three irrigations, solvent iv) indicated that there were two spots corresponding to raffinose and melibiose. Enzymic hydrolysis (invertase) gave a mixture containing fructose and

only one spot identical with melibiose. Paper chromatographic examination of the acid hydrolysate (as 4b) showed that glucose, galactose and fructose were present. There was insufficient material for a quantitative estimation.

6c. This fraction was shown (solvent iv, three irrigations) to contain two spots which travelled at about the disaccharide rate. Examination of the acid hydrolysate on a paper chromatogram (solvent ii) showed that only glucose was produced.

Fraction 7. After elution from the papers only 4 fractions contained sufficient carbohydrate for further examination. (Acid hydrolyses were as for 4b).

7a. The material was very slow-travelling (solvent iv) - slower than raffinose. Chromatographic examination (solvent ii) of the acid hydrolysate showed that glucose was the major constituent, accompanied by galactose and a trace of xylose.

7b. This ran slightly in front of raffinose on the chromatogram (solvent iv). Chromatographic examination (solvent ii) of the acid hydrolysate showed galactose and glucose (ratio ca. 2:1 by visual estimation) present in addition to small amounts of arabinose and xylose.

7c. This spot travelled (solvent iv) at approximately the rate of a disaccharide. It was unchanged by invertase action and was shown to contain glucose and xylose (after acid hydrolysis). The proportion of glucose was greater than that of xylose.

7d. The spot travelled very slightly faster than 7c on the chromatogram (solvent iv) but in other respects was similar.

The polysaccharides of lucerne.

N.B. All evaporations were carried out at 30-35°C. under reduced pressure unless stated otherwise.

Hot water extractions.

(A) A portion (10 gm.) was extracted overnight with 80% ethanol in a Soxhlet apparatus to remove mono-, di- and oligosaccharides. The extracted residue was boiled with water (250 cc.) for 3 hours under reflux and filtered while hot. The residue was thoroughly washed with water and the extract and washings were combined. The solution gave a blue colour with iodine.

An aliquot (150 cc.) of the extract (320 cc.) was acidified (10 cc., 16.5N H_2SO_4) and boiled under reflux for 4 hours. The ratio of sugars in the hydrolysate, determined after paper chromatographic separation (solvent ii) and quantitative analysis, was galactose (4.7 parts by weight), glucose (5.4 parts), arabinose (9.6 parts) and xylose (1.0 part).

(B) Lucerne (135 gm.) was extracted overnight in two Soxhlet thimbles with 80% ethanol. The alcohol extracted residue was boiled under reflux with water (750 cc.) for 3 hours. The filtered extract and washings were concentrated to 1000 cc. Aliquots from this were used in the following experiments.

1) A portion (1 cc.) was evaporated to dryness in a weighed dish placed on a water bath at 60°C. A current of air was played

on the surface. The dish + residue were finally dried in a desiccator over P_2O_5 (in vacuo). The weight of the residue was 0.0129 gm., i.e. total solids in extract = 12.9 gm./1000 cc.

2) An aliquot (5 cc.) was carefully evaporated in a silica crucible (as in 1) and ignited to constant weight over a Meker burner. The ash content was 4.0 gm./1000 cc.

3) The total nitrogen content of the hot water extract represented 2.3% of the total solids (by micro-Kjeldahl estimation on 5 cc. aliquot). After clarification with cadmium sulphate and barium hydroxide solutions, the nitrogen content was 0.8% of the total solids i.e. clarification reduced the soluble nitrogen content considerably.

Ammonium sulphate fractionations.

4) An aliquot (100 cc.) of the extract was used for an ammonium sulphate fractionation (109). This was unsuccessful; no precipitates were obtained.

5) A portion (300 cc.) of the extract was clarified [cadmium sulphate (25 cc.; 0.36N) and barium hydroxide (equimolecular volume)] and filtered. The volume of the extract was reduced to 50 cc. and an ammonium sulphate fractionation attempted.

Precipitates were obtained at 30%, 40%, 50% and 70% salt concentrations, but no fractionation of the polysaccharides was obtained. The fractions (examined by paper chromatography after hydrolysis) all contained a mixture of galactose, glucose, arabinose and xylose as well as traces of rhamnose.

N.B. i) The ammonium sulphate solutions were stirred for 15 minutes to allow equilibration after addition of fresh portions of the salt.

ii) The fractions were separated by centrifuging.

iii) Acid hydrolysates (1N acid for 4 hours under reflux) were neutralised with $BaCO_3$ and the solutions examined on chromatograms irrigated with solvent ii.

6) An aliquot (550 cc.) was concentrated to 30 cc. before a fractionation was attempted.

The solution yielded fractions at 30, 50 and 70% salt concentrations. No fractionation was evident, the same five sugars being present in each product viz. galactose (2 parts), glucose (2), arabinose (4), xylose (1) and rhamnose (traces). [Estimated by visual examination of the stains with aniline oxalate.]

Cold water extraction.

A sample (52.4 gm.) was extracted with 80% ethanol overnight in a Soxhlet apparatus. The residue was shaken for 6 hours with water (600 cc.) at room temperature. The insoluble residue was filtered and re-extracted overnight. The extracts and washings were combined and concentrated to 200 cc. No colour was observed with iodine. The solution was clarified (as p. 33) and concentrated to 100 cc. The total solids (p. 138) were 2.78 gm. An ammonium sulphate fractionation was unsuccessful.

The residue after cold water extraction was boiled for 3 hours with water (600 cc.) under reflux. The extract and washings were

concentrated to 150 cc. and clarified. An ammonium sulphate fractionation of this extract was also unsuccessful.

Copper complex formation from a hot water extract.

Alcohol-extracted lucerne (10 gm.) was boiled under reflux (3 hours) with water (100 cc.). The extract and washings were concentrated to 20 cc. Sodium hydroxide (5 cc.; 5N) was added followed by 25 cc. of freshly prepared Fehling's solution. The solution was allowed to stand overnight but no insoluble copper complex was formed.

Ammonium oxalate extractions.

A large sample of milled lucerne (1000 gm.) was extracted overnight with 80% ethanol in a Soxhlet apparatus. This was the starting material used for all the oxalate and alkali extraction experiments.

1) A sample (10 gm.) was extracted with two portions (150 cc. each) of 0.5% w/v ammonium oxalate on a boiling water bath for two periods of $1\frac{1}{2}$ hours each. The combined extracts and washings (350 ml.) had pH 8.0. An aliquot (50 cc.) was acidified (3.25 cc.; 16.5N H_2SO_4) and boiled under reflux for 4 hours. The solution was neutralised (32) with sodium hydroxide (4N) using methyl red as indicator and evaporated to 30 cc. The salts were precipitated with ethanol (150 cc., 95%) and filtered. The filtrate was concentrated and examined on a chromatogram irrigated with solvent iii. The sugars

present were galactose, arabinose (in quantity), glucose and xylose (in small quantity) and rhamnose (trace). The presence of a uronic acid was shown by spraying a duplicate chromatogram with naphtho-resorcinol-hydrochloric acid solution.

The residue from the oxalate extract was shaken overnight with water (250 cc.) and filtered. The filtrate and washings were evaporated to 50 cc. (35°C., reduced pressure). The solution was made 1N with respect to sulphuric acid and hydrolysed by boiling under reflux for 4 hours. The hydrolysate was neutralised and examined on a chromatogram (as above). The sugars present were galactose, glucose, arabinose and xylose. By visual examination the ratio was 2:1:2:1. No mannose was detected.

After extraction with cold water, the residue was boiled with water (250 cc.) for 3 hours. The insoluble material was filtered off and washed with hot water. The extract and washings were evaporated to 50 cc. The solution was made normal with respect to sulphuric acid and boiled under reflux for 4 hours. The hydrolysate was examined in the same way as that of the cold water extract above. The sugars present were xylose, arabinose, glucose and traces of galactose.

2) The alcohol-extracted material (10 gm.) was treated with oxalate (as in 1 above) for three two-hour periods. From the combined extracts and washings (600 cc.), an aliquot (300 cc.) was concentrated to 40 cc. Of this, 20 cc. were made 1N w.r.t. sulphuric acid and boiled under reflux for 4 hours. After neutralisation (as in 1) the monosaccharides were separated on a chromatogram irrigated with

solvent iii for 40 hours. The same sugars as in the first oxalate extract were detected. There was also a uronic acid present.

The oxalate-extracted residue was extracted with cold and hot water as in (1) above. The sugars found in both hydrolysates were galactose and arabinose mainly accompanied by glucose and xylose.

The monosaccharides in the oxalate extracts (1) and (2) were separated on duplicate chromatograms (solvent iii), eluted and estimated. The results are given in the table.

Sugar	2 x 1½ hour		3 x 2 hour	
	Wt. on paper(mg.)	Ratio	Wt. on paper(mg.)	Ratio
Galactose	0.535	4.35	0.761	7.93
Arabinose	0.448	3.64	0.748	7.99
Glucose	0.096	0.78	0.218	2.25
Xylose	0.123	1.0	0.096	1.0
Rhamnose	trace	-	0.019	0.20

They show that by prolonging the extraction with ammonium oxalate it was possible to remove larger quantities of the galactose and arabinose material but there was also an increase in the amount of glucose extracted simultaneously.

3) Alcohol-extracted lucerne (10 gm.) was treated with 0.5% ammonium oxalate (2 x 250 cc.) for two 1½ hour periods on a boiling water bath.

The two extracts were filtered hot and the residues washed with hot water. The filtrates and washings were combined and dialysed (using cellophane dialysis tubing) against running water to remove salts (140 hour).

The oxalate extract had become gelatinous and the gel was centrifuged down (2500 r.p.m. for 30 minutes) to give (i) a gel and (ii) a solution which was discarded. The gel was dissolved in 0.5% ammonium oxalate and two volumes of acetone were slowly added to the vigorously stirred solution. The precipitate was centrifuged down, washed with ethanol of increasing concentration and dried over P_2O_5 (in vacuo) to give a dark brown powder.

Analysis of the isolated product (ca. 300 mg.).

Crude protein 6.01 %

Ash 11.47 %

Carbohydrate. 21.0 mg. was hydrolysed (4 hours boiling under reflux) with 1N H_2SO_4 (10 cc.). After cooling the solution, ribose (17.7 mg.) was added as a reference sugar. Galactose, arabinose and ribose were estimated after chromatographic separation (in solvent ii). The amount of polygalactose (i.e. galactose in the polysaccharide form) was 0.54 mg. and of polyarabinose 0.42 mg., i.e. 0.96 mg. or 4.57% carbohydrate. The amounts of the other sugars (glucose, xylose) were very small and were only detected by examination of the aniline oxalate-sprayed strips under ultra violet light.

"Pectin". The total uronic anhydride content (85) was 74.2%.

4) A sample (25 gm.) of alcohol-extracted lucerne was shaken for 20 hours with 0.5% ammonium oxalate (300 cc.). The insoluble material was centrifuged down and separated. The centrifugate was dialysed [as in 3] and the gel and liquor separated by centrifuging. Acetone (700 cc.) was slowly added to the solution (350 cc.) and the precipitate isolated (as in 3).

Analysis of the isolated product (ca. 500 mg.).

Crude protein 13.4%

Ash 5.3%

Carbohydrate. 19.0 mg. was hydrolysed (as above). Ribose (17.2 mg.) was added as a reference sugar. The amounts of poly-galactose and polyarabinose were 1.04 mg. and 0.42 mg. respectively, i.e. 1.46 mg. or 7.68% carbohydrate.

"Pectin". The total uronic anhydride content (85) was 67.9%.

Sodium hydroxide extractions.

(1) Alcohol-extracted lucerne (p. 141) (20 gm.) was shaken with sodium hydroxide (800 cc.; 4% w/v) for 24 hours. The insoluble material was centrifuged down and washed with NaOH (4%). The combined solution and washings (1200 cc.) were acidified (glacial acetic acid 200 cc.) to pH 4-5 and allowed to stand 24 hours before centrifuging and separating the precipitate. The supernatant was retained for the preparation of a xylose rich fraction.

The acid-insoluble precipitate was dissolved as completely as possible in 4% NaOH and a small amount of insoluble material was discarded. The reprecipitation with acetic acid was repeated twice, the final precipitate being allowed to stand overnight in the acidified solution. The product was washed with ethanol of increasing concentration and dry ether and finally dried over P_2O_5 (in vacuo). The product was pale grey - U.1.

A crude xylan was precipitated from the acidified solution by dropwise addition of two volumes of acetone and separated by centrifuging. The polysaccharide was redissolved in NaOH (4%) and purified by copper complex formation (23). The purification was repeated twice, the final product being dried (as U1). This gave a product X1.

Analysis of U1 and X1.

U1. (a) A sample (20.0 mg.) was hydrolysed by boiling for 4 hours under reflux with 1N H_2SO_4 (20cc.). The total reducing power of the hydrolysate (neutralised with 4N KOH) was equivalent to 7.90 ml. of 0.005N sodium thiosulphate. Since xylose was found to be the main sugar present, the Somogyi factor 0.130 was used to express the weight of sugar i.e. the 20 mg. sample contained 1.03 mg. reducing sugar (as xylose) or 0.91 mg. pentosan. This corresponded to 4.6% of the sample.

(b) A sample (31.0 mg.) contained nitrogen equivalent to 22.33 mg. crude protein i.e. 72.0%.

(c) Ash accounted for 4.4%.

(d) A uronic acid was present but not estimated (see p. 147).

X1. (a) A sample (33.0 mg.) was hydrolysed (as U1(a)) with 20 cc. H_2SO_4. The hydrolysate was neutralised and diluted to 50 ml. and the total reducing value of a 5 ml. aliquot determined. This was 17.63 ml. of thiosulphate i.e. 176.3 ml. for the whole hydrolysate. Calculated as xylose (shown to be the main constituent) this represents 22.92 mg. sugar or 20.17 mg. pentosan i.e. 61.1% of the sample.

(b) A sample (34.0 mg.) contained nitrogen equivalent to 0.31 mg. crude protein i.e. 0.9%.

(c) Ash accounted for 5.1%.

(d) A uronic acid was present but not estimated.

Naphthoresorcinol test for uronic acids (133).

To 5 ml. of the solution containing the uronic acid (or a suspension of the material containing the acid) was added 1 cc. of naphthoresorcinal solution (1% in absolute ethanol) and 6 cc. of HCl (conc.). The solution was heated carefully to boiling point over a micro-burner and maintained at that temperature for 1 minute. The dark solution was set aside for 4 minutes and when cool, ether (12 cc.) was added and the liquids mixed. The ether layer showed a blue colour if a uronic acid was present. For comparison, tests using water and a monosaccharide (glucose) were carried out.

Isolation of a xylan from lucerne.

A large sample of oven-dried milled lucerne was extracted with 80% ethanol overnight in a large Soxhlet apparatus. The residue was oven-dried at 80°C. and shaken with NaOH (4% w/v) for 24 hours at room temperature. The extraction was done in five batches during which 400 gm. of the alcohol-extracted lucerne was treated. The acid-insoluble material was discarded after centrifuging. The xylan was precipitated by addition of two volumes of acetone and separated at the centrifuge. The acetone-insoluble material was dissolved in 400 cc. of NaOH (4%), a small quantity of insoluble material being discarded.

Fehlings solution (450 cc.) was poured into the solution and the gel allowed to stand overnight. The complex was centrifuged down, separated and decomposed with HCl (250 cc.; 2N). An equal volume of acetone was added gradually to the solution to precipitate the polysaccharide. This was separated by centrifuging and washed with 300 cc. "wash solution" (400 cc. 95% ethanol; 50 cc. 2N HCl; 50 cc. H_2O). The precipitate was dissolved in NaOH (4%) and the purification repeated a further 4 times.

The final product was washed with (a) wash solution (3 x 300 cc.), (b) ethanol of increasing concentration and (c) anhydrous ether. The white product was dried over P_2O_5 (in vacuo) - yield 6½ gm. of xylan.

Experiments carried out on the Xylan.

1) Attempts were made to remove the polysaccharides containing the other sugars found in the preparation.

(a) A small portion of the xylan was shaken for 24 hours with cold water at room temperature. The insoluble material was centrifuged down, washed thoroughly with water and hydrolysed (5 hours boiling under reflux with 1N H_2SO_4). Examination of the hydrolysate showed the xylan still contained the arabinose, rhamnose and the hexose.

(b) An attempt using boiling water (two three-hour periods under reflux) was also unsuccessful.

(c) The xylan was soluble in 0.1% ammonium oxalate but insoluble in 0.001%. A portion of the xylan was shaken with 0.001% oxalate at room temperature (as in (a)). No separation was achieved.

2) The xylan contained 0.25% crude protein.

3) A sample of the xylan (218.1 mg.) was hydrolysed for $5\frac{1}{2}$ hours under reflux with 1N H_2SO_4 (15 cc.). After cooling, ribose (27.7 mg.) was added and after neutralisation of the hydrolysate, the sugars were separated on two pairs of replicate chromatograms run in solvents i and ii for 40 hours. The glucose + galactose (combined), arabinose, xylose and ribose were all estimated from the papers run in solvent ii, any overlapping of xylose and ribose being corrected by the ribose value found from the papers run in solvent i.

Rhamnose was also estimated from these papers.

The results are shown in the table.

	ml. of 0.005N thiosulphate	wt. of sugar on paper(mg.)	Calculated to ribose = 100%	% of the xylan
Galactose) Glucose)	0.09 (factor 0.140)	0.013	1.6	0.73
Arabinose	0.08	0.012	1.4	0.64
Xylose	10.05	1.367	163.3	74.87
Ribose	1.14	0.204	-	-
Rhamnose	0.22	0.037	4.5	2.06

Notes. (i) The amount of arabinose, rhamnose and glucose + galactose were so small that the duplicate sugar zones were eluted together and the titration obtained by the Somogyi estimation divided by two before being tabulated.

(ii) The Somogyi factor calculated for rhamnose hydrate was 0.188 and from the weight thus obtained the weight of rhamnose (in polysaccharide form) was calculated.

4) The xylan contained 2.59% ash.

5) The total uronic anhydride content was 12.54%.

These analyses account for 93.68% of the product.

Extraction of starch from lucerne leaves.

The alcohol-immersed leaf (p. 127) was macerated and extracted with 90% ethanol in a beaker (at boiling point) five times, the extracts at this stage being colourless. This residue was used in the following experiments.

1) A sample (25 gm.) was shaken overnight with water (500 cc.). The residue was filtered and the extraction repeated twice. The filtrates were combined and concentrated (30°C.; reduced pressure). The solution was brought to density 1.07 by addition of sodium chloride (volume of solution 500 cc.). Toluene (30 cc.) was added and the solution vigorously shaken for two hours. The mixture was centrifuged and the precipitate collected. Examination under a microscope showed this to contain particles which stained blue with iodine. The amount of isolated product was very small.

2) The method used in a second preparation was that described for tobacco leaves (108). The sample (30 gm.), of alcohol-extracted lucerne leaves, was macerated (5 minutes) with 0.02M phosphate buffer pH 7.0 (300 cc.) and toluene (25 cc.). The residue was filtered through muslin and the extraction repeated three times. The combined extracts were centrifuged and the solid material separated and washed with methanol. The product was air-dried. The following analyses were carried out.

i) Moisture content (including methanol ?) - determined by drying over P_2O_5 15.1%

ii) Crude protein content 28.6%

iii) Ash content 18.7%

iv) Carbohydrate. A portion (79.4 mg.) was hydrolysed (1.5N H_2SO_4; 10cc.) by boiling under reflux for 4 hours. Ribose (26.0 mg.) was added as a reference sugar and the solution neutralised ($BaCO_3$) and spotted onto chromatograms irrigated with solvents i and ii. The results showed

Hexose (mainly glucose, only a trace of galactose) 30.6%

Arabinose 3.2%

Xylose 1.4%

These analyses account for 97.6% of the sample weight.

v) Enzymic hydrolysis. The isolated product stained blue with iodine. A spatula-tip of the material was placed in a small test-tube with a few crystals of sodium chloride. Saliva (0.5 cc.) was added and the digest left at room temperature. After 5 hours, no stain was observed with iodine, and paper chromatographic examination of the solution showed the presence of maltose and glucose.

Occurrence of the Uronic Acids in lucerne.

Extraction of the pectin. A sample (50 gm.) of the alcohol-extracted lucerne (p. 141) was boiled for 3 hours with water under reflux. The insoluble residue was filtered and thoroughly washed with water, and a small portion (ca. ½ gm.) ground in a tube with hot (60°C.) water (3 cc.) and a small amount of sand.

The extract was filtered and a drop of iodine added to it. A strong blue colour was observed.

The boiling water extraction was repeated twice until an extract which did not give a blue colour with iodine was obtained after grinding. The residue (sample 1) was dried overnight in an oven at 80°C. The uronic anhydride content was 11.20%.

A portion (1.788 gm.) of sample 1 was extracted with ammonium oxalate (0.5%; 55 cc.) for 3 hours on a boiling water bath. The residue was filtered, washed thoroughly with oxalate, water and finally acetone. The oven-dried (80°C.) residue (sample 2) weighed 1.618 gm. i.e. the loss in weight during the oxalate extraction was 9.5%. The uronic anhydride content was 3.13%.

The percentage of the uronic anhydride material extracted was calculated,

Sample 1, 1.788 gm. containing 11.20% uronic anhydride i.e. 200.3 mg.
Sample 2, 1.618 gm. " 3.13% " " i.e. 50.6mg.
i.e. the uronic anhydride material extracted = 149.7 mg. or 74.8% of the total amount present. This indicates that the major part of the uronic acid content is in the form of pectin.

Identity of the uronic acids in lucerne.

1) Uronic acid in the ammonium oxalate extract. The fraction Cii prepared during the examination of the experimental conditions for the routine analysis of lucerne (p. 69), was examined. No purification

of the crude ethanol-insoluble product was attempted. The large weight of the product indicated probable coprecipitation of other material with the pectin and polysaccharides. The uronic anhydride content of the crude material was 18.71%.

Hydrolysis of the crude product. Hydrolysis of a sample (100 mg.) of the crude product (20 cc.; 1N H_2SO_4; 5 hours boiling under reflux) and examination on a paper chromatogram irrigated with solvent ii (40 hours) showed the presence of galactose, arabinose and small amounts of glucose, xylose and rhamnose accompanying the uronic acid material. The distance travelled by the acid (on a paper in solvent i) was identical with that of a sample of galacturonic acid. This distance was very similar to that moved by glucuronic acid but this latter acid can be distinguished by the spot due to the lactone (glucurone) which travelled very much more rapidly than the free acid. There was no evidence of this lactone spot in the hydrolysate.

Oxidation with Br_2-HBr. A sample (750 mg.) of the product was oxidised in HBr (75 cc.; 7.5%) containing Br_2 (3.5 cc.) for 10 hours on a water bath under reflux (129). Further additions of Br_2 (3.5 cc. each) were made 4 hours and 7 hours after heating began. On cooling a small amount of crystalline material settled out of the solution.

This was filtered off and recrystallised (hot water) to give

a white crystalline product m.p. 215°C. (decomp.), mixed m.p. with mucic acid 213-214°C. (decomp.). The original uronic acid was therefore galacturonic acid.

The HBr solution was neutralised (K_2CO_3) and acidified with glacial acetic acid. No potassium hydrogen saccharate separated indicating glucuronic acid was not present in the original sample.

2) Uronic acid precipitated from the 1N H_2SO_4 extract. On cooling, the 1N H_2SO_4 extracts of lucerne leaf and stem samples deposited a white product. This material (from ca. 10 seasonal variation samples in 1955) was centrifuged down and separated. The product was washed with alcohol of increasing concentrations and finally with anhydrous ether, and dried over P_2O_5 (in vacuo).

A sodium fusion test showed the absence of nitrogen, sulphur and halogens. Hydrolysis (6½ hours boiling under reflux with 20 cc. 1N H_2SO_4) of a sample (170 mg.) and paper chromatographic examination of the hydrolysate (in solvents i and ii) showed the absence of any monosaccharides, the single spot being due to the presence of a uronic acid at the distance travelled by galacturonic acid. There was no evidence of unhydrolysed material at the starting line. A uronic anhydride estimation accounted for 77.99% of the product. The ash content was 9.7%.

Oxidation with Br_2-HBr. A sample (160 mg.) was oxidised in HBr (16 cc.; 7.5%) with Bromine (0.8cc.) on a boiling water bath under

reflux for 10 hours. Further additions of Br_2 (0.5 cc. each) were made after 4 hours and 7 hours.

A small amount of crystalline material which separated on cooling the reaction mixture, was recrystallised from boiling water. The white crystals had m.p. 212°C. (decomp.). A mixed m.p. with mucic acid was 210-212°C., and a mixed m.p. with a sample of the product from 1 (above) was 211-212°C. (both with decomp.). As in 1, no potassium hydrogen saccharate was obtained.

3) Uronic acid present in the xylan.

Oxidation with Br_2-HBr. A sample (1.010 gm.) of the xylan (uronic anhydride content 12.5%) was oxidised in HBr (16 cc.; 7.5%) with bromine (0.8 cc.) on a boiling water bath for 10 hours. Further additions (0.8 cc.) of bromine were made after 4 hours and 7 hours heating.

A small amount of insoluble material was separated (by forceps) from the hot reaction mixture but no crystals separated on cooling and concentration to a small volume (5 cc.), i.e. mucic acid was not produced and therefore galacturonic acid was not present.

Preparation of aldobiuronic acid. A portion (1.215 gm.) of the xylan was hydrolysed under reflux with oxalic acid (3% w/v; 70 cc.) for 12 hours on a boiling water bath. The solution was cooled, neutralised ($BaCO_3$), filtered and the precipitate washed. The filtrate was concentrated to 30 cc. and methanol (250 cc.; dried over

magnesium turnings) was added to the stirred solution. The precipitate was filtered, washed with dry methanol and dried over P_2O_5 (in vacuo).

A small quantity (0.164 gm.) of the product (0.356 gm.) was hydrolysed with sulphuric acid (0.5N.; 16 cc.) for 6 hours on a boiling water bath under reflux. The hydrolysate, after cooling, was neutralised ($BaCO_3$) and the barium sulphate filtered and washed. The barium salt of the aldobiuronic acid was precipitated with dry methanol (50 cc.) after concentration of the filtrate and washings to 5 cc.

A spatula tip of the precipitate was dissolved in 1 cc. warm (25-30°C.) 2N sulphuric acid and hydrolysed for 20 hours on a boiling water bath under reflux. The hydrolysate was cooled, neutralised ($BaCO_3$) and the insoluble salts separated by filtration. The filtrate was evaporated to dryness and the sugars dissolved in 3-4 drops of hot dry methanol and spotted onto a chromatogram irrigated with solvent ii. The only sugar present was xylose. The barium salt of the acid was dissolved in a few drops of water, shaken with Amberlite resin IR 100 to remove cations and spotted onto a chromatogram. The paper (irrigated with solvent i) showed the presence of xylose (trace) and a uronic acid.

Reduction of the aldobiuronic acid. A portion (5 mg.) of the barium salt of the aldobiuronic acid was dissolved in methanolic hydrochloric acid (1%; 5 cc.) and boiled under reflux (6 hours) to form the methyl ester. The solution was cooled, neutralised (Ag_2CO_3) and the excess silver ions removed as the silver sulphide. The solution was evaporated to dryness and dissolved in water (5 cc.). Potassium borohydride (15 mg.) was added and the solution was shaken overnight. The mixture was acidified with 3 drops glacial acetic acid, and the ions were removed by shaking (½ hour) with Amberlite resins (IR 4B and IR 100). Methanol (50 cc.) was added and the solution evaporated to dryness to remove the methyl borate. The residue was dissolved in 1N hydrochloric acid (5 cc.) and hydrolysed for 4 hours under reflux on a boiling water bath. The solution was neutralised ($Ag._2CO_3$), the excess silver ions being removed as silver sulphide, and after concentration, the sugars were spotted onto a chromatogram irrigated with solvent ii. The paper was sprayed with aniline oxalate solution and the presence of xylose and glucose shown, therefore the aldobiuronic acid isolated from the xylan contained glucuronic acid and xylose.

PART 2. DISCUSSION

The carbohydrates of lucerne.

The methods used for the analysis of lucerne (and ryegrass) gave no indications of the actual carbohydrates present in the plant. The monosaccharides and sucrose were the only constituents estimated directly. Analysis of the oligosaccharides and polysaccharides has only given information about the proportions of each constituent monosaccharide and not about the composition of the individual polysaccharides.

In order to obtain information about these various carbohydrates, a series of experiments was carried out. These may be divided into four sections. The oligosaccharides were separated and tentatively identified by chromatographic and hydrolysis experiments; the polysaccharide material was treated with water, ammonium oxalate and sodium hydroxide and the extracts examined for the soluble carbohydrates; the presence of a "starch-like" fraction was demonstrated and the occurrence and identity of uronic acids were studied.

The oligosaccharides. In view of the difference found in the nature of the reserve carbohydrates viz. starch and fructosan in lucerne and perennial ryegrass, it is of interest to compare the composition of the oligosaccharide fraction from these two plants.

In perennial ryegrass, the oligosaccharides present in the 80% ethanol extracts are mainly short chain fructosans (62) but small amounts of galactose-containing compounds are also found and some

pentose oligosaccharides. Lucerne does not contain fructosan and therefore fructosan oligosaccharides are unlikely to be present.

It was found that oligosaccharides could be separated by adsorption on a mixture of charcoal and supercel and elution with aqueous ethanol. A disadvantage in using charcoal columns is that the adsorbing power is high and may lead to incomplete recoveries from the column. Better recoveries and equally good separations have been reported using a column of charcoal and celite which had been pre-treated with stearic acid (4).

The mixture of sugars from an 80% ethanol extract of lucerne was clarified and deionised, and an attempt was made to separate the oligosaccharides on a charcoal-supercel column. Mechanical changing of the solvent was unsatisfactory, and in a second attempted fraction- ation (on a column pretreated with stearic acid) gradient elution was used giving a slightly better separation. Mixtures of oligo-saccharides were still obtained and it was necessary to separate these by paper chromatography.

The presence of a readily hydrolysed sucrose link in the oligo-saccharide molecule can be used for its characterisation. For example, if a trisaccharide is present and is found to produce fructose and a disaccharide which can be identified, this provides information regarding the structure of the molecule.

A test experiment on sucrose, raffinose and stachyose showed that the hydrolysis of the sucrose link could be carried out by invertase on the paper strip (12). There was no evidence of poly-merisation of fructose under the conditions used.

Using ascending solvent chromatography and multiple development, the extreme complexity of the oligosaccharide fractions obtained from

the stearic acid treated column was evident. It was found that after six irrigations, fraction 5 had been resolved into twelve separate spots, some only in very small quantity. Because of the complex mixtures obtained, it was decided that each of the four main fractions containing oligosaccharides (Nos.4,5,6 and 7, p.131) should be further separated on paper chromatograms.

Six ascending solvent irrigations and extraction of the sugars gave the following fractions.

[* Chromatographically similar to specimen sample.]

	Approx. size of molecule. (estimated by rate of flow)	Constituent sugars	Possible identity
4a	Probably larger than tetrasaccharide	Insufficient for analysis	Not known
4b	tetrasaccharide	Glucose	Maltotetrose(?)
4c	disaccharide	Glucose(1 part) Galactose(1 part)	Melibiose*
4d	disaccharide(s)	Glucose, galactose, arabinose, xylose	Mixture of more than one oligosaccharide
5a	tetrasaccharide	Galactose(2) Glucose(1) Fructose(1)	Stachyose*
	trisaccharide	Galactose(1) Glucose(1) Fructose(1)	Raffinose*
5b	disaccharide	Galactose(1) Glucose(1)	Melibiose*
5c	disaccharide	Glucose	Maltose*
	disaccharide	Glucose, Xylose	Not known
5d	disaccharide	Glucose, Fructose	Sucrose*
5e	monosaccharide	Insufficient for analysis	Not known
5f	monosaccharide		
5g	ran faster than monosaccharide		

(continued overleaf)

	Approx. size of molecule. (estimated by rate of flow)	Constituent sugars	Possible identity
6a	tetrasaccharide	Galactose(2) Glucose(1) Fructose(1)	Stachyose* + higher homologue (?)
	trisaccharide) overlapping on	Galactose(1) Glucose(1) Fructose(1)	Raffinose*
6b	trisaccharide) chromatogram	Fructose, Melibiose	Raffinose*
	disaccharide	Melibiose	Melibiose*
6c	disaccharide	Glucose	Not known
	disaccharide	Glucose	Not known
7a	tetrasaccharide	Glucose,Galactose,Xylose	Not known
7b	trisaccharide	Galactose(2) Glucose(1) Arabinose, Xylose (small amounts)	Not known
7c	disaccharide	Glucose, Xylose	Not known
7d	disaccharide	Glucose, Xylose	Not known

The results show the complex mixture of oligosaccharides found in the lucerne extract.

The major constituents were raffinose, stachyose, melibiose, i.e. members of the raffinose family (52). In one fraction (6a) the ratio galactose:glucose:fructose for one component was 2.20:1:0.73. This high galactose value may be due to admixture with a small amount of the higher homologue, verbascose, which contains 3 galactose units in the molecule.

Since these experiments were carried out on the alcohol extract from

oven-dried lucerne, it cannot be claimed that these oligosaccharides all occur naturally in the fresh plant. For example, melibiose may have been produced by loss of a fructose unit from raffinose. Similarly the glucose-containing disaccharide, corresponding chromatographically to maltose, may have been produced from the starch during the drying process. The identities of the other disaccharides which apparently contained only glucose were not known.

It is evident, therefore, that the oligosaccharides present in lucerne are mainly those of the raffinose family, sucrose, raffinose, stachyose and possibly verbascose. In addition there are glucose-containing oligosaccharides and those containing pentose sugars which may be intermediaries in the hemicellulose synthesis or may have been removed from the polysaccharides during the drying of the sample.

The polysaccharides. The alcohol-extracted lucerne was treated with a number of solvents to obtain information about the polysaccharides actually present and to isolate the pure polysaccharides.

Some polysaccharide material was extracted with hot water and probably included some starch. On hydrolysis, it yielded galactose, glucose, arabinose, xylose in the approximate ratio (5:5:10:1). An attempt to separate this mixture into individual polysaccharides by fractionation with ammonium sulphate (109) was unsuccessful. The fractionation was tried before and after

clarification of the solution and at different carbohydrate concentrations but no enrichment of any fractions was evident.

Cold water extraction did not remove any particular polysaccharide preferentially and no material giving a colour with iodine was detected. The cold water removed a similar mixture of polysaccharides (in smaller yield) to the hot.

Fehlings solution frequently forms an insoluble complex with some of the hemicellulose fractions of various plants, but in the case of the hot-water-soluble polysaccharides of lucerne, no fractionation could be obtained in this way.

Removal of pectin by extraction with ammonium oxalate has been common for many years and since the galactose- and arabinose-containing material was known to be closely associated with the pectic substances, it was thought that this solvent might remove these polysaccharides. This was in fact the case. Extraction of the material with 0.5% ammonium oxalate gave a solution rich in galactose and arabinose and containing a uronic acid although glucose and xylose were also present. Not all the galactose- and arabinose-containing material could be removed since the insoluble residue gave, on cold water extraction, quantities of galactose and arabinose material and even in a subsequent hot water extraction these sugars were shown to be present. It was noted that if the ammonium oxalate extraction was prolonged, a greater proportion of glucose-containing polysaccharide was simultaneously separated, and very small quantities of rhamnose could be detected.

Attempts were made to fractionate the oxalate extracts. Removal of inorganic salts by dialysis against running water caused the separation of some material in gel form, which was separated as completely as possible from the solution. Examination of this gel after hydrolysis showed it to contain arabinose, galactose and a uronic acid as well as small quantities of glucose and xylose. Treatment of the liquor with acetone gave a product of similar composition to the gel. It appeared therefore that although no satisfactory separation of any pure araban, galactan or arabogalactan was achieved during these experiments, it was possible to prepare from the oxalate extracts a galactose- and arabinose-rich fraction. The proportion of polysaccharide was very small (ca. 5%) in both the sample prepared from the gel and from the solution. Pectin accounted for a large percentage of each fraction (ca. 70-75%).

A 4% sodium hydroxide extract of lucerne gave on acidification some insoluble polysaccharide material which was shown to contain xylose, glucose and a uronic acid accompanied by a small quantity of arabinose. This crude product contained much nitrogen indicating probable protein contamination (ca. 72%) and contained only 5% carbohydrate (mainly xylose).

The acidified solution gave on addition of acetone a product rich in xylose. This was not a pure xylan, since it was shown to contain a uronic acid and also glucose, galactose and arabinose as well as a very small amount of rhamnose. No mannose was detected in the alkali extract.

After these experiments, a xylan fraction was prepared by alkali extraction. Following several precipitations as a copper complex (with Fehlings solution), it was analysed and found to contain

Glucose + galactose	0.73% dry weight
Arabinose	0.64
Rhamnose	2.06
Xylose	74.87
Uronic anhydride	12.54 (shown to be glucuronic acid, p. 158)
Crude protein	0.25
Ash	2.59

Attempts to separate the other polysaccharides from the xylan were unsuccessful.

The starch in lucerne. The failure to detect any starch in the cold water extractions (described on p. 140) may have been due to the very small quantities present in the material examined which had been gathered at the end of the season. [From the seasonal variation study the amounts present at that date would be small.]

On shaking a sample of lucerne leaf (gathered early in the season) with water, a small quantity of material (stained blue with iodine) was obtained. By extracting with buffer (pH 7.0) a crude starch was obtained. This was found to contain ca. 30% glucose and was contaminated with protein (30%), ash (20%) and other polysaccharides (5%). Hydrolysis of the crude product with salivary

amylase removed the iodine staining power and a paper chromatogram of the digest showed it to contain maltose and glucose.

Laidlaw and Reid did not detect starch in a cold water extract of clover (82), but Hardwick during a seasonal study of clover found material which stained with iodine in almost all samples. These contained polysaccharide material which was hydrolysed (enzymically) under conditions recommended for starch estimations.

It is probable that the starch material in lucerne was similar to this clover polysaccharide in view of the difficulty in extracting it. The failure to detect it on occasions may be due to the small quantities present at certain times of the season.

The uronic acids in lucerne. It was noted from the literature that uronic acids (in combined form) had been found in lucerne. As seen from the seasonal study (1955), the total uronic anhydride content of the lucerne was ca. 5%-10% of the dry weight.

Extraction of the lucerne with ammonium oxalate showed that 75% of the uronic anhydride material was removed. This indicated that pectin was the main source of the plant's uronic anhydride and the presence of galacturonic acid in the material extracted by ammonium oxalate was confirmed by the isolation of mucic acid after oxidation with bromine.

During the study of the seasonal variations, a white material was found to precipitate from the normal sulphuric acid extracts of both leaf and stem samples of lucerne on cooling. This material

was isolated and shown to be very rich in uronic anhydride (ca. 80%). No monosaccharides were detected after acid hydrolysis. By bromine oxidation to mucic acid, the constituent uronic acid was shown to be galacturonic acid and the isolated product itself is probably pectin although it may be partially degraded (during extraction) when compared with the pectin occurring naturally in the lucerne.

Glucuronic acid was reported to be present in the 4% sodium hydroxide extract of lucerne roots (19) but the uronic acid in the hemicellulose portions of the aerial parts was claimed to be a methyl ether of glucuronic acid (106). The evidence for the presence of the ether group was based upon a small methoxyl content found in the barium salt.

The xylan isolated from lucerne contained a uronic acid which accounted for ca. 12% of the dry weight. Oxidation of the xylan with bromine did not yield any mucic acid; the acid was therefore not galacturonic acid.

By partial hydrolysis of the xylan with dilute oxalic and dilute sulphuric acids, an aldobiuronic acid was isolated and the sugar present was shown to be xylose. Reduction of the aldobiuronic acid with potassium borohydride and hydrolysis of the disaccharide showed that only xylose and glucose were present. The original uronic acid was therefore glucuronic acid and not a methyl ether derivative. The uronic acid unit would appear to be directly connected to the xylan chain and not to any of the other polysaccharides isolated with the xylan.

Summary.

1) Methods have been developed for the analysis of the carbohydrates in the aerial portions and the roots of perennial ryegrass and in the leaves and stems of lucerne, and were applied to samples gathered at intervals during the year.

2) The mono-, di- and oligosaccharides were extracted with aqueous ethanol, the fructosan (in the ryegrass) with cold water, and estimated. The other plant polysaccharides were estimated after a two-stage sulphuric acid hydrolysis.

3) In perennial ryegrass (in 1953) no major trends were found in the amount of glucose, fructose or sucrose, but there was a gradual increase in the proportion of cell-wall polysaccharides. The fructosan functioned as a reserve polysaccharide and there was a relation between the quantity present and the growth of the grass. A maximum content of 21% of the dry weight of the stem was followed by a decrease in the Autumn.
The aftermath growth had a moderately high fructosan content/(17% in the stem) but the proportion of cell-wall polysaccharides was lower than in the first growth.

4) In 1955, the study of perennial ryegrass was extended to include the roots. The amount of water-soluble carbohydrate in the roots depended upon the supply from photosynthesis (i.e. on the amount of sunshine), and the demands for the production of new

tissue both above and below ground level. In the Autumn, the fluctuations in the root followed the graph showing the amount of sunshine, no very large carbohydrate reserves being detected. The proportion of cell-wall polysaccharides in the roots and in the aerial portions increased with maturity.

5) Lucerne was studied during two consecutive summers (1954, 1955). The plant grew taller in the second year (30 cf. 18 inches) and reached constant height more rapidly than in the first. After constant height was reached, growth continued by increase in stem weight resulting in very woody stems at the end of the 1955 season.

6) The changes in the carbohydrate constituents of lucerne have been shown to be dependent on the rate and stage of growth of the plant and upon the weather conditions.
In the first year of growth, the amount of ethanol-soluble sugar depends upon the weather conditions during the plant's growth. In the second year, the stage of growth controlled the carbohydrate composition to a much greater extent than the sunshine.
There was an obvious similarity in the changes in the amount of hexose, sucrose and glucosan (i.e. starch) in 1955, and it has been proposed that the changes in these constituents represent the balance of the carbohydrate which has been photosynthesised over the amount required for the plant's metabolism (i.e. respiration, growth requirements and the development of flower and seed).

7) The variations in the polysaccharide fractions (except starch), reflect the change from the very soft young plant to the woody stems found at the end of the second year. There is an increasing cellulose and lignin content and in addition, the xylan content of the polysaccharides shows an increase with age.

8) By comparison of the results from the study of perennial ryegrass and lucerne, it is seen that the general nature of the carbohydrates is similar in these mono- and di-cotyledons, although the reserve polysaccharides (i.e. fructosan and starch), and the proportions of the individual carbohydrates, are different.

9) An examination of the carbohydrates present in lucerne was undertaken.

The oligosaccharides consisted mainly of members of the raffinose family, viz. raffinose, stachyose and possibly also verbascose. Other molecules which were found (e.g. maltose and melibiose) may not occur naturally in the lucerne but were possibly produced during the drying of the material examined.

Arabinose- and galactose-containing polysaccharide material could be extracted (along with the pectin) by ammonium oxalate and a xylan fraction was prepared from a sodium hydroxide extract. This was not a pure xylan (75% xylan) and it contained glucuronic acid (12%) and other carbohydrates (3%). A "starch-like" material was extracted from fresh lucerne leaves with phosphate buffer solution (pH 7). The crude product which was isolated contained glucose(30%) and gave a blue colour with iodine. Hydrolysis with saliva, produced maltose and glucose and the products did not give a stain with iodine.

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Analytical Studies on the Carbohydrates of Grasses and Clovers. VIII. Changes in Carbohydrate Composition during the Growth of Perennial Ryegrass.

By D.J. Mackenzie and Clare B. Wylam.

Analyses of the carbohydrates in the leaf and stem of perennial ryegrass were carried out during uninterrupted growth from May to November. Although there were individual variations in the amounts of glucose, fructose and sucrose present, no major seasonal trends in these sugars were evident. The plant accumulated in the stem a reserve of fructosan which reached a maximum of 21% of the dry matter before it diminished in the autumn. There was a gradual increase in the percentages of cell-wall polysaccharides. The relation between fructosan accumulation and synthesis of cell-wall material during the growth of the plant is discussed.

The autumn aftermath growth was analysed and its composition compared with that of the first growth.

Introduction.

The importance of the water-soluble constituents of herbage has been recognised for a considerable time, both from the point of view of its energy value in fresh and preserved feedingstuffs, and on account of the advantage of a high sugar content in silage making. In the latter respect, an approximate knowledge of the fructosan content of a grass is particularly useful, since this polysaccharide

undergoes a wide variation throughout the summer and is probably sufficient under certain conditions to contribute most of the sugar necessary for adequate lactic acid production.[1]

Production of fatty acids by fermentation of cellulose is well known as a source of energy in ruminants,[2,3,4] and the importance of hemicelluloses in the diet is now also being realised.[5] For instance Heald[6] has recently suggested that in 24 hours 60-80 g. xylan may be fermented in the rumen of a sheep at pasture.

In view of the recognition of the importance of these constituents it is essential to obtain information about the variation of both the water-soluble and cell-wall carbohydrates throughout the growing season.

Considerable studies have been made of the composition of different grasses, and the distribution of the water-soluble carbohydrates in the plant.[7-15] The major water-soluble constituent is usually fructosan, which is stored in the stem, and goes through a peak about the time of full emergence of the head.[11-13] Waite and Boyd[16] have found that in some grasses fructosan accumulation is temporarily interrupted by a slight fall when the growing point changes from the vegetative to the floral state. Waite (private communication) has made a comparison of grasses with different growth characteristics which suggests an inverse relation between rate of growth and fructosan accumulation. Experiments have shown an increase of cellulose, pentosan, and lignin which reaches a maximum in the mature plant.[11,17-20] Cutting has been found to reduce the water-soluble carbohydrates[21,22] and cellulose[23] in grass.

It has been shown in this laboratory that changes in carbohydrate composition may occur in the fresh plant after cutting[24] or during the drying process.[25] Oven-dried samples have been used frequently in the past but on analysis these may give results which do not represent the true composition of the fresh plant.

Previous analytical methods have been based on determination of the reducing sugars in alcohol and water extracts before and after acid hydrolysis. In some cases fructose has been estimated after oxidation of glucose in mixtures of the two sugars. One disadvantage of this method is the determination of small quantities of reducing substances other than sugars. A more serious error can be caused by the expression of oligosaccharides as sucrose.

Very little accurate work has been carried out on the analysis of the cell-wall polysaccharides of grasses. The main method which has been used is a delignification treatment[11,26] to leave a cellulosic residue in which the remaining pentosan is estimated from the yield of furfuraldehyde on treatment with hydrochloric acid.[11,27] Considerable losses of hemicellulose may be incurred during the delignification and there may be interference by other substances in the pentosan estimation.

New techniques using quantitative paper chromatography have been developed in this laboratory for the individual determination of the free sugar and polysaccharide components of grasses.[28-30] By the application of these methods to the analysis of samples immersed in hot alcohol immediately after cutting, results have been obtained

which represent as closely as possible the composition of the living plant.

Perennial ryegrass was selected as a suitable grass in which to study carbohydrate changes since it is of considerable agricultural importance, being used extensively throughout the British Isles for grazing, hay and silage.

Experimental.

The grass used for the experiment was pure S24 strain of Lolium perenne, sown in rows 1 ft. apart in a $\frac{1}{20}$ acre plot on 23rd March, 1953, at Boghall, Midlothian. The soil, pH 6.2, of medium potash and high phosphate content, had an application of farmyard manure in the early Spring, followed by 22 lb. potato manure (9%N; 9% P_2O_5; 15% K_2O) at the time of sowing. It received no further manurial treatment during the growth of the grass.

The plot was divided into 108 squares, seven of which were selected at random for each cutting, in order to obtain a representative sample. For the analysis of the first growth no square was cut twice. The first sample was cut on 18th May, 1953, when the grass had reached a height of $2\frac{1}{2}$ inches. The stage of growth was followed by measuring the average height and determining the relative weights of dry leaf and stem in the plant.[16,31] On 10th August, when the grass had reached the hay stage, 35 previously unclipped squares were cut back and the aftermath allowed to grow without further cutting. Samples from seven squares of this second

growth were analysed from September to November for comparison with the first growth.

The grass was cut at a height of ½ - 1 inch above ground level, the material from the seven squares thoroughly mixed and a sub-sample taken for analysis. The plant was separated into leaf and stem, the leaf blade being removed at the ligule, so that the stem sample included the leaf sheath as well as the culm. The flower head was discarded except on 7th September when the head was removed and the spikelets analysed.

In order to minimise any error due to diurnal variation in the water-soluble carbohydrates, samples were always collected at 10 a.m. Experiment has shown that little or no change takes places before this time. For instance, the fructosan content of perennial ryegrass cut at intervals during a sunny day in March did not change during the morning, rose to a maximum during the early afternoon and fell off sharply at sunset. At 6.30 a.m., 12.15 p.m., 3.45 p.m., and 7.10 p.m., the fructosan content was respectively 2.5%, 2.6%, 3.1% and 1.5% of the dry weight of the grass (cf. also Waite and Boyd[16]).

The time taken for sampling and dissection was about 30 minutes, after which the separated parts were weighed and immersed in hot alcohol. The sugars in an 80% ethanol extract were determined after paper chromatographic separation and the fructosan determined in a cold water extract.[28,29] The hemicelluloses and cellulose in the ethanol- and water-extracted residue were analysed by a two-stage

sulphuric acid hydrolysis, followed by paper chromatographic separation and estimation of the sugars in the hydrolysates.[30] The hemicelluloses are expressed as polygalactose, polyarabinose and polyxylose. The chemical structure of these polysaccharides is unknown, but by comparison with the cell-wall composition of other members of the Gramineae[32-5] it is probable that many of the polysaccharides contain more than one sugar residue. The lignin figures are derived from an acid-insoluble residue and do not represent values of pure lignin,[30] but for comparative purposes they indicate the trend of lignification. Moisture contents were determined by oven-drying at 80°C. and crude protein (N x 6.25) was determined in the oven-dried material by the micro-Kieldahl method. The leaf-stem ratio was determined on a dry weight basis by dissecting a representative sample of 100 tillers and oven-drying.

The individual analytical results are presented in table II and figures 3-8, and are expressed as percentages of the dry matter of that part of the plant analysed. Where leaf and stem were analysed separately, results for the whole plant can be calculated from the leaf-stem ratio.

Weather records were obtained from the Meteorological Sub-Station at Boghall, Midlothian, and the data are presented in table I and figure 2.

Results and Discussion.

Effect of sunshine on the water-soluble sugar content.

The sunshine received by the plant just before cutting has a

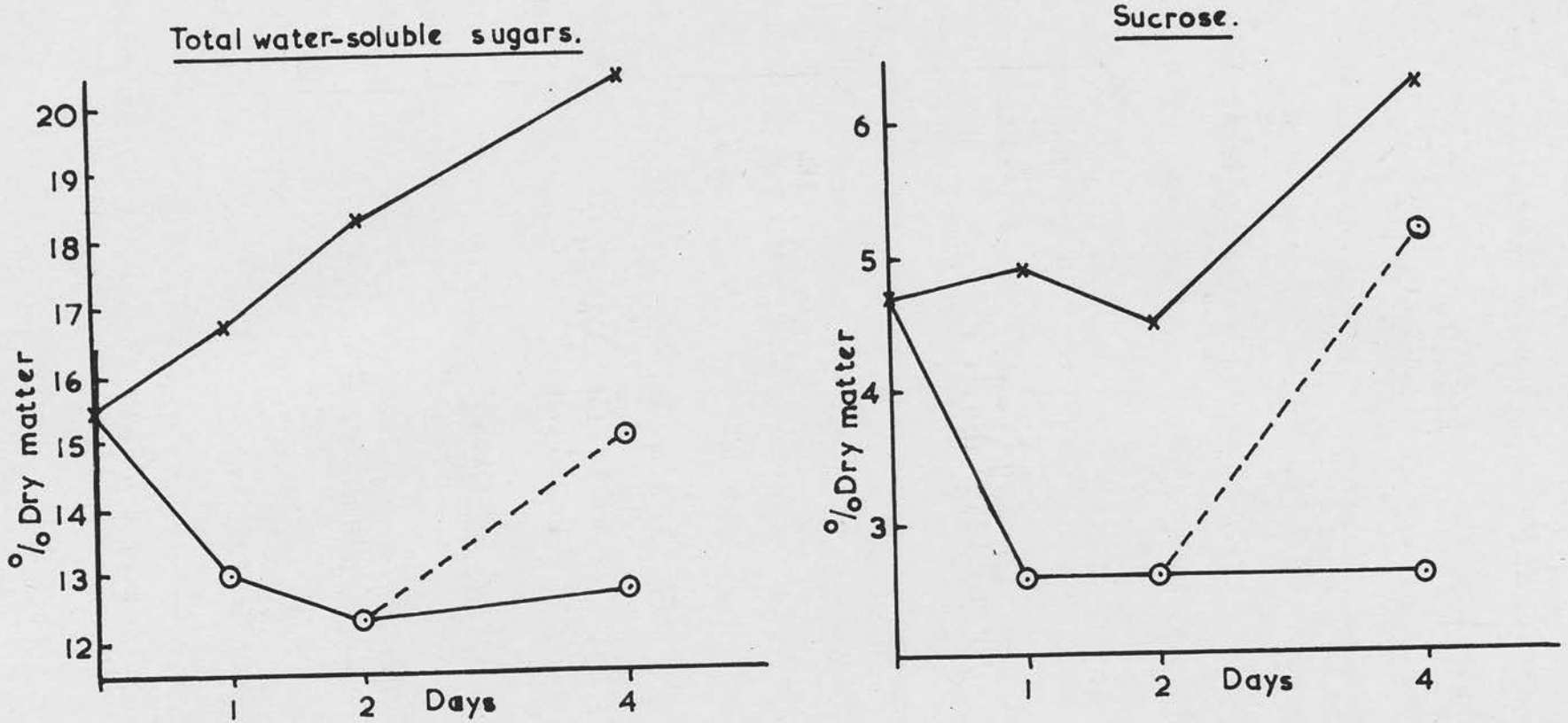


FIG.1. EFFECT OF SUNSHINE ON SUGARS.

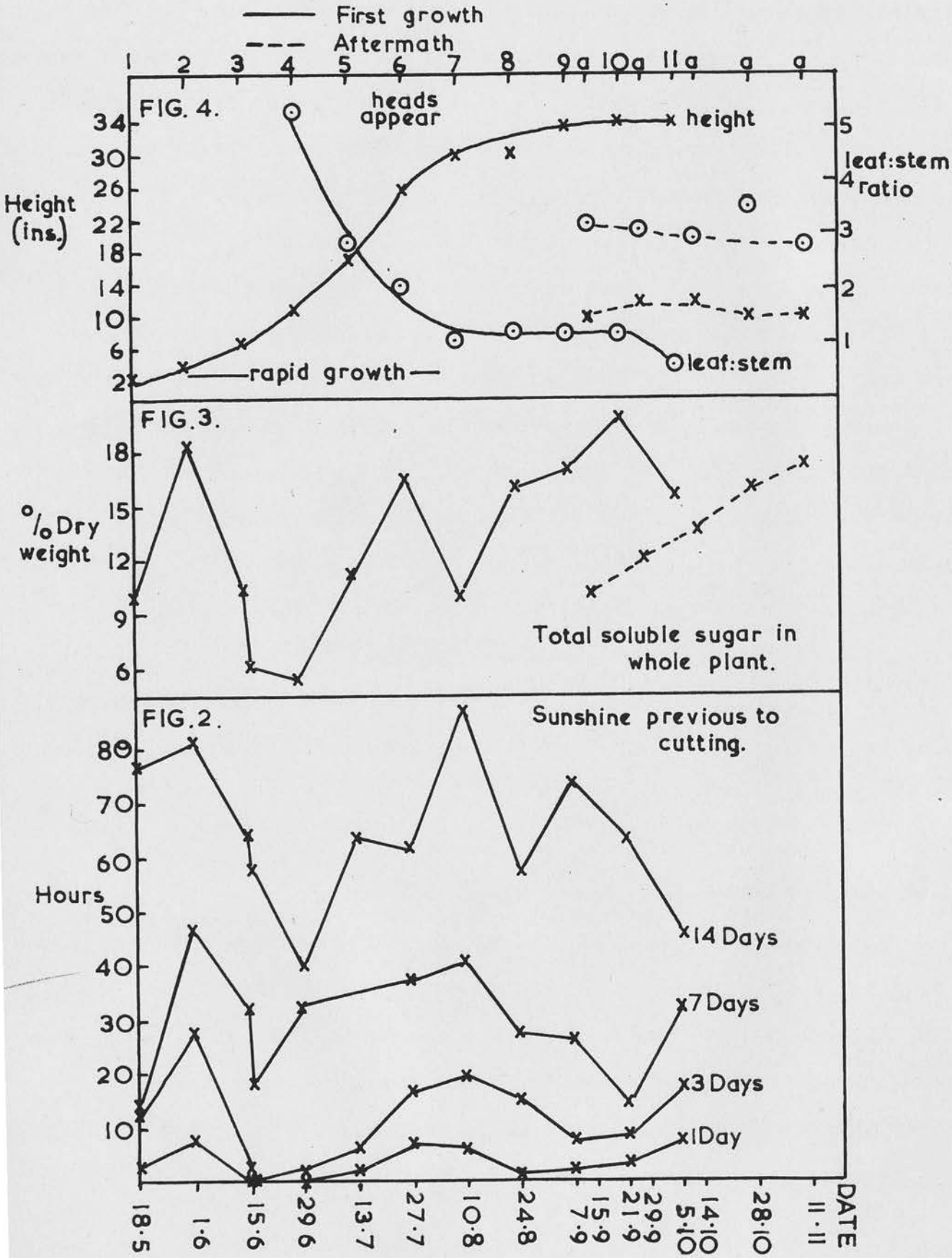
×——× Uncovered plot.
⊙——⊙ Covered plot.
⊙- - -⊙ Plot uncovered on 2nd day.

marked effect on its water-soluble sugar content. One half of a plot of young perennial ryegrass in the vegetative state was covered with hessian, giving lighting conditions comparable to daylight on a dull day, whilst the other half received full sunlight. The changes in the total sugars and sucrose are shown in figure 1. After four days there was a difference in the total sugars of 7.7% of the dry matter, due mainly to a sudden fall in the sucrose content during the first day after covering, whereafter the latter remained constant. Half of the covered plot was uncovered on the second day of the experiment. The sucrose content then rose sharply during the next two days. The fructosan contents of the covered and uncovered plots were both 7.2% after one day, but were respectively 6.5% and 8.2% after two days. There was no further change in the fructosan content of the covered plot after four days. This can be correlated with the lack of sucrose for synthesis.

It is important therefore to relate compositional changes with sunshine as well as other factors when interpreting seasonal changes in the plant.

Climatic conditions and growth of the grass.

The monthly temperature and rainfall figures for 1953 are given in table I. There was a period of very poor weather from 12th to 29th June when there was very little sunshine. Figure 2 shows the sunshine on the day before cutting and the total sunshine in 3, 7 and 14 days previous to cutting. It is impossible to say how far back


First growth
Aftermath
1 2 3 4 5 6 7 8 9a 10a 11a a a
FIG. 4.
heads appear
height
leaf:stem ratio
Height (ins.)
34 30 26 22 18 14 10 6 2
5 4 3 2 1
rapid growth
leaf:stem
FIG. 3.
% Dry weight
18 15 12 9 6
Total soluble sugar in whole plant.
FIG. 2.
Sunshine previous to cutting.
Hours
80 70 60 50 40 30 20 10
14 Days
7 Days
3 Days
1 Day
18·5 1·6 15·6 29·6 13·7 27·7 10·8 24·8 7·9 15·9 21·9 29·9 5·10 14·10 28·10 11·11 DATE

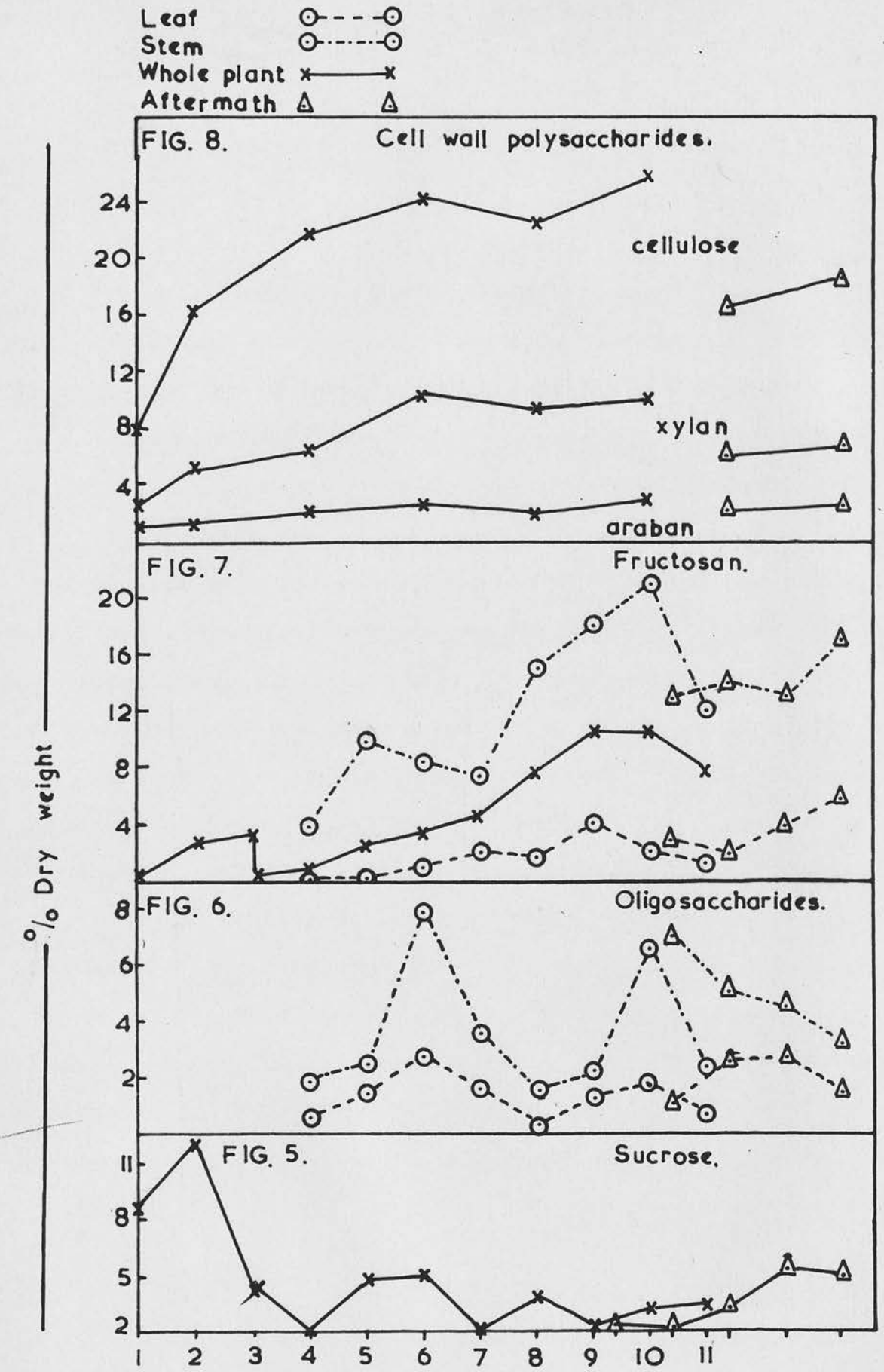
the sunshine-photosynthesis relationship should be traced, but it is obvious that there were two samples which had received maximal amounts of sunshine, namely those cut on 1st June and 10th August. In these samples a high sugar content would be expected, as was the case on 1st June (figure 3). Nevertheless, on 10th August the total soluble sugar content was relatively low (figure 3) and therefore at this stage of development another factor must be predominant.

The plant, initially very leafy, underwent a period of rapid growth by production of more stem, as shown by the decrease in leaf:stem ratio and increase in height (figure 4). By 10th August the height was almost at a maximum and the leaf:stem ratio had become constant. The initial rapid growth took place during the period of little sunshine, so that a depletion of sucrose and fructosans is expected at this stage. There was indeed a marked decrease of sucrose, oligosaccharides and fructosan between 1st and 17th June (table II), causing the total soluble sugar to fall from 18.6% to 6.2% (figure 3). The first heads appeared about 27th July.

The aftermath differs from the first growth in that it reached the asymptotic stage of growth while still very short and before it was long enough to be divided into leaf and stem (figure 4). The leaf:stem ratio was higher than that of the first growth, indicating the vegetative nature of the aftermath, which was observed to remain short and leafy throughout the whole period. It did not flower.

Compositional changes in the first growth.

The analytical results are presented in table II.


Leaf
Stem
Whole plant
Aftermath
FIG. 8.
Cell wall polysaccharides.
cellulose
xylan
araban
FIG. 7.
Fructosan.
FIG. 6.
Oligosaccharides.
FIG. 5.
Sucrose.
% Dry weight
1 2 3 4 5 6 7 8 9 10 11

The hexoses did not follow any obvious seasonal trends. In the leaf the combined glucose and fructose content remained at a fairly constant level of 1-2%, whereas in the stem it was always higher, fluctuating between 3% and 7%. The leaf generally contained more fructose than glucose, whereas in the stem the reverse was the case.

There were only slight differences between the sucrose contents of the leaf and stem. When the sucrose in the whole plant (figure 5) is correlated with the sunshine on the day before cutting (figure 2) it is seen that the sucrose peaks on 1st June and 27th July coincide with maximum amounts of sunshine. In view of this, and the obvious influence of day to day sunlight shown above, there is no evidence in these results for any definite seasonal pattern in the variation of this sugar. The sucrose content of the plant at any particular time is probably dependent on a great many factors, including the rate of photosynthesis, stage of growth, and demands made on sucrose or its precursors for synthesis of larger molecules. Waite and Boyd[16] claim that the sucrose content increases with stage of growth and goes through a seasonal peak, but they have not taken sunlight into consideration. It is also possible that some fructosan oligosaccharides were estimated as sucrose by Waite and Boyd's method.

Chemical studies of the mixture of oligosaccharides in 80% ethanol extracts of S24 perennial ryegrass have shown that these are mainly short-chain fructosans.[29,36] Small quantities of galactose-containing compounds are also present, including melibiose and

raffinose[37] and sometimes traces of pentose oligosaccharides. The oligosaccharide mixture from the samples was separated from the hexoses and sucrose on thick paper chromatograms and hydrolysed. Estimation showed a wide variation in the relative proportions of fructose, glucose and galactose in the hydrolysate but the major sugar was usually fructose. The percentage of total oligosaccharides was always lower in the leaf than in the stem, and it passed through two peaks (figure 6), but the significance of this is difficult to interpret. The general pattern resembles that of the sunshine on the day before cutting, and the peak on 27th July coincides with a peak in the sucrose content. On the other hand, the peak on 21st September coincides with the date of maximum fructosan content in the stem (figure 7). It is possible therefore that the oligosaccharide content also is a reflection of the rate of photosynthesis, depending upon the amount of sucrose available and the rate of fructosan synthesis at that time.

The fructosan content of the leaf was never greater than 4%, but that of the stem was much higher (figure 7), and followed the general pattern observed by Waite and Boyd[16] for timothy, cocksfoot and meadow fescue grasses. There was a gradual build up of fructosan in the young plant, followed by a very rapid drop between 15th and 17th June, due probably to bad weather and little photosynthesis, with a resulting drain on the existing fructosan. There was a subsequent increase in stem fructosan, followed by a fall between 13th July and 10th August. It has already been seen that

there was a lot of sunshine in the fortnight before 10th August and that the low fructosan content on that date was unexpected. Since the first flower heads appeared about 27th July, the growing points in the tillers which were to bear flowers must have changed from the vegetative to the floral state at, or just before, this time. It is therefore likely that a depletion of fructosan took place to satisfy energy requirements for flower initiation. Waite and Boyd have not observed a fall in fructosan at this stage in S23 perennial ryegrass, but this could have been due to good weather conditions and a rate of synthesis more than adequate for energy requirements. S23 is a slow-growing ryegrass, building up in the stem stores of fructosan greater than in the quicker-growing S24 strain.

On the other hand, it is obvious from figure 4 that the plant was growing very rapidly between 13th July and 10th August, and since these results are expressed on a percentage basis, it is possible that this fall in fructosan is only an <u>apparent</u> one due to the production of new structural tissue. There may be no actual depletion of the fructosan but only a temporary arresting of its synthesis while its photosynthetic precursors are deflected into the path of cell-wall polysaccharide synthesis. This would be evident if the results were expressed in the form of yield per unit area, but this was not calculated on account of the difficulty of determining the yield accurately on a small scale. After 10th August, when the period of rapid growth had terminated, the stem fructosan increased rapidly until it reached the maximum value of 21% on 21st September. The subsequent fall is likely to be due

mainly to translocation followed by transformation to starch in the seed.[15,16] The withering of some of the leaves in the autumn and the consequent reduced rate of photosynthesis is another cause of depletion of the reserve.

Changes in the cell-wall constituents followed the same pattern in both leaf and stem and are shown for the whole plant in figure 8. Polygalactose remained less than 1% and polyarabinose increased from 1% to 3% with no appreciable difference between leaf and stem. There was a gradual increase of cellulose and polyxylose and these were both higher in the stem than in the leaf. Lignin also increased as the plant matured. A slower rate of increase in the percentages of the cell-wall polysaccharides is obvious after 27th July, and since the plant stopped growing at about this time there would be little increase in the overall amount of these constituents thereafter. It was at this time that the fructosan started to increase again.

Thus during the period of rapid growth the emphasis was on synthesis of cell-wall polysaccharides, but after 27th July synthesis of cellulose and pentosan was considerably reduced and, instead, reserves of fructosan began to be accumulated in the stem. It is clear therefore that fructosan is a fluctuating reserve polysaccharide, stored in the stem, and represents the balance of photosynthetic products after the plant's requirements for energy and synthesis of protein and cell-wall polysaccharides have been satisfied.

Analysis of the seeds was carried out on 7th September. The contents of free glucose and free fructose were relatively low, but

the sucrose content was of the same order as that in the leaf and stem. The oligosaccharide content was relatively high, whereas the fructosan was extremely low. Starch (2.8%) was present and was determined in a hot water extract.[29] The polyarabinose and cellulose contents were of the same order as those in the stem but the polyxylose (14.7%) was relatively higher.

The Aftermath Growth.

The lack of active growth of the aftermath is reflected in the relatively low contents of cell-wall polysaccharides, these being in the region of 18% cellulose and 6% polyxylose compared with about 24% and 10% respectively in the first growth. The first growth and aftermath had approximately the same height and leaf:stem ratio on 13th July and 15th September respectively. On these dates the soluble sugar contents of the whole plants were almost the same but the subsequent patterns were different. The hexose, sucrose and oligosaccharide contents were in the same range as those of the first growth. The stem fructosan was in the region of that found in the mature first growth after 10th August, and had risen to 17% by 11th November. However, this high content in the stem would not contribute a great deal to the average content of the whole plant since the leaf:stem ratio was very high.

Waite and Boyd[22] found low values for fructosan when S23 perennial ryegrass was cut every time it reached a height of 8-10 inches. However, the fructosan content was slightly higher in the

autumn when the growing point remained in the vegetative state and more time had elapsed between cuttings due to slower growth. In the present experiment the aftermath reached a height of 10 inches before it was analysed on 15th September, and was subsequently allowed to grow without further cutting. Since it was too late in the season for formation of flower heads, and since little further growth or synthesis of cell-wall polysaccharides occurred, the plant had an opportunity to build up a reserve of fructosan in the same way as did the first growth when other demands were low.

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Table I. Monthly Temperatures and Rainfall, 1953.

	May	June	July	Aug.	Sept.	Oct.	Nov.
Aver. temp. (°F.):	60.4	62.6	63.0	64.6	60.7	53.5	49.3
Rainfall (mm.):	57.6	78.1	102.7	44.3	76.5	31.6	108.3

Table II. Water-soluble and cell-wall carbohydrates in grass as % of the dry matter.

Date	Part of Plant	L/S Ratio	Crude Protein	Glucose	Fructose	Sucrose	Oligo-saccharides	Fructosan	Poly-galactose	Poly-arabinose	Poly-xylose	Cellulose	"Lignin"
18 May	L + S		29.1	0.4	0.4	8.5		0.6	0.2	1.1	2.7	8.0	0.6
1 June	L + S		25.2	0.7	0.7	12.1	2.5	2.5	0.3	1.3	5.2	16.5	2.1
15 June	L + S		25.6	0.4	2.3	3.2	1.4	3.3					
17 June	L + S		27.2	0.2	0.8	3.5	1.1	0.6					
29 June	Leaf	5.3	26.6	0.4	1.0	2.2	0.6	0.4	0.6	2.0	6.2	21.0	4.1
	Stem		16.6	1.7	2.1	1.9	1.9	3.8	0.7	2.2	7.0	23.5	1.2
13 July	Leaf	2.9	22.6	0.6	1.2	3.4	1.5	0.2					
	Stem		13.5	3.7	2.8	4.9	2.5	9.9					
27 July	Leaf	2.1	18.2	0.7	1.3	5.7	2.8	1.0	1.0	2.8	9.8	24.4	3.4
	Stem		10.0	4.6	2.3	4.2	8.0	8.4	0.5	2.7	12.2	24.2	3.1
10 Aug.	Leaf	1.1	15.9	0.8	1.1	3.0	1.7	2.0					
	Stem		8.7	1.8	0.9	1.2	3.7	7.4					
24 Aug.	Leaf	1.2	16.7	0.4	0.7	3.8	0.4	1.8	0.6	2.0	7.6	20.5	3.4
	Stem		8.6	4.0	2.4	4.3	1.6	15.1	0.8	2.1	11.6	25.2	6.2
7 Sept.	Leaf	1.2	10.6	0.3	0.8	2.8	1.4	4.1					
	Stem		5.5	2.2	1.3	2.4	2.3	18.2					
	Seed		10.3	0.6	0.8	2.1	4.1	0.7					
21 Sept.	Leaf	1.2	13.4	0.5	0.7	3.6	1.8	2.1	0.7	2.8	9.3	24.7	3.4
	Stem		6.3	1.5	1.4	3.2	6.7	21.0	0.8	3.5	13.3	27.5	5.8
5 Oct.	Leaf	0.6	15.9	0.5	0.8	4.6	0.8	1.3					
	Stem		6.8	1.5	1.1	3.1	2.5	12.0					
AFTERMATH													
15 Sept.	L + S	3.2	14.8	0.4	0.5	2.5	4.9	2.0					
29 Sept.	Leaf	3.1	20.7	0.2	0.5	1.6	1.2	3.0					
	Stem		10.9	2.3	2.8	4.6	7.1	13.0					
14 Oct.	Leaf	2.9	13.7	0.7	1.1	3.4	2.7	2.0	0.7	1.9	5.3	16.2	3.0
	Stem		9.7	1.2	1.2	4.0	5.1	14.0	0.9	2.8	8.3	18.2	2.4
28 Oct.	Leaf	3.5	16.4	0.3	0.6	6.1	2.8	4.0					
	Stem		9.4	1.1	1.2	4.0	4.6	13.0					
11 Nov.	Leaf	2.8	16.5	0.3	0.6	5.1	1.6	6.0	0.9	2.3	6.4	18.9	4.8
	Stem		9.3	1.1	1.4	5.0	3.3	17.0	1.1	3.0	8.5	18.9	4.2

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